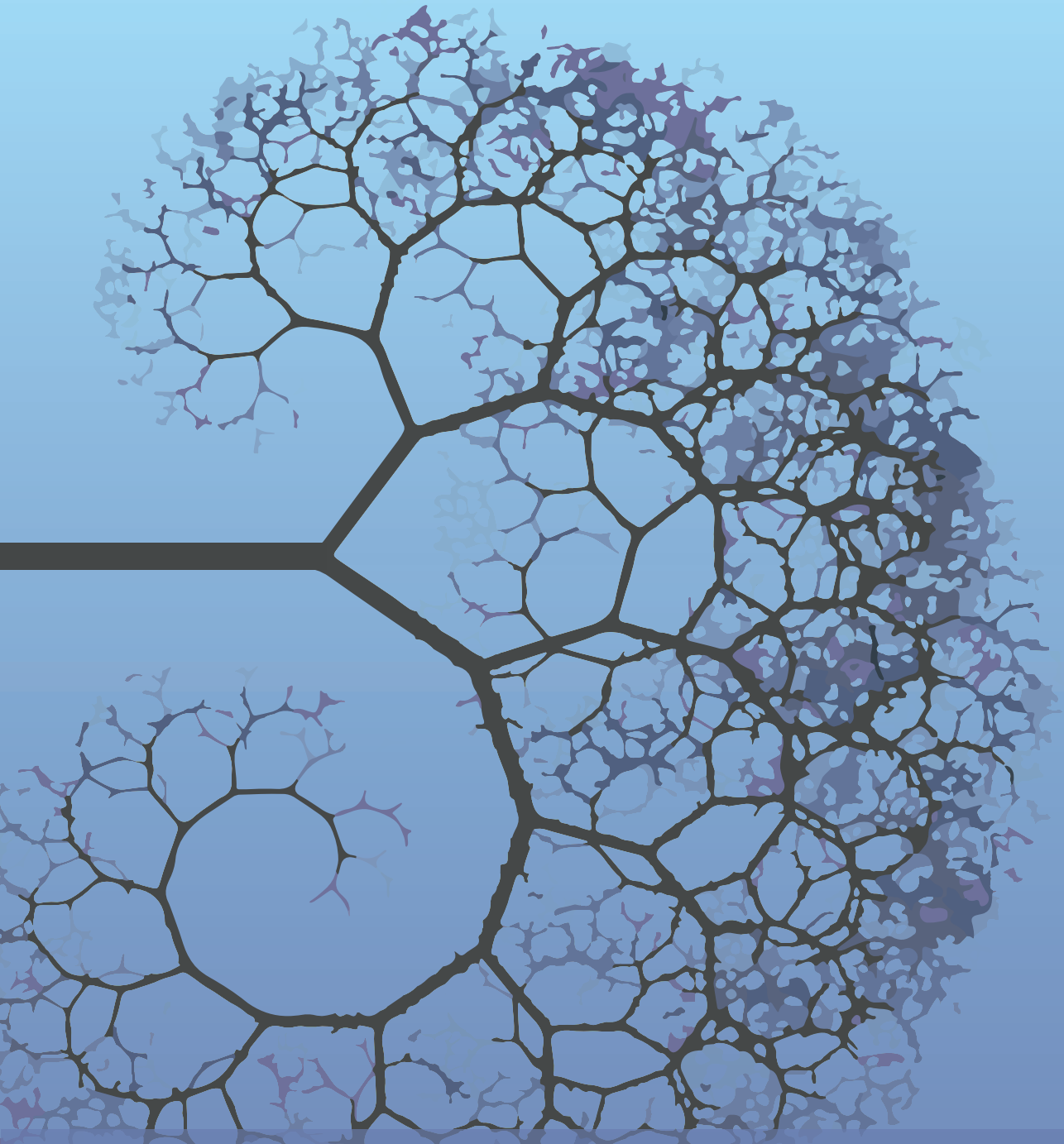

TOWARDS A BIOARTIFICIAL KIDNEY

Insights in uptake and elimination of cationic solutes by proximal tubule epithelial cells

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Insights in uptake and elimination of cationic solutes by proximal tubule epithelial cells

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Carolien Monica Stephanie Schophuizen
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Promotoren	Prof. dr. L.P.W.J. van den Heuvel (KU Leuven)
	Prof. dr. R. Masereeuw (Universiteit Utrecht)
	Prof. dr. J.G.J Hoenderop
Manuscriptcommissie	Prof. dr. W.F.J. Feitz
	Prof. dr. J.A. Jansen
	Dr. J.I. Rotmans (Leiden Universitair Medisch Centrum)

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General introduction

HISTORY OF RENAL FAILURE

Although renal failure is a disease as old as humanity itself, our ability to understand kidney functioning, and the origin of this life threatening disease is much more recent. Around 350 BC, the Greek philosopher and scientist Aristotle thought of the bladder as the main urine producing organ. Approximately four centuries later, Galen of Pergamos was the first to propose that urine is produced when blood is cleared by the kidneys [1].

The term uremia (Greek for urine poisoning, or "urine in the blood") did not develop until the isolation of urea from urine in the 18th century. The two French physicians that led the discovery, Dr. Fourcroy and Dr. Vauquelin, predicted that if urea is not "separated" from the blood, an excess might lead to specific disorders [2]. At the time, this hypothesis was not easily accepted [3]. However, when in 1856 Picard developed a reproducible and sensitive method for the measurement of blood urea, the theory behind uremia gained popularity [4,5]. Since then, innovative research in the field of nephrology, has led to a dramatic improvement in the treatment of chronic kidney disease (CKD). Nowadays, the preferred treatment of patients who suffer from end stage renal disease (ESRD) involves the transplantation of a donor kidney. However, due to the shortage of donor kidneys, most patients undergo renal replacement therapy such as dialysis. This procedure, based on physical processes like osmosis and diffusion, removes accumulating solutes such as urea from the circulation. Dr. Graham (1805–1869), considered a founding father of dialysis therapy, was one of the first to describe that crystalline urea could be dialyzed from urine through semi-permeable membranes [6]. In 1945, Dr. Kolff introduced the first clinically functional hemodialysis machine for the treatment of uremia [7,8]. This device consisted of a rotating drum kidney in a static open bath, with 20 meters of cellophane dialysis tubing wound around it (Figure 1.1). Over the last 70 years, thorough optimization of the semi-permeable material used in these dialysis devices, and optimization of the design, have made the therapy much more efficient, compact and safe [9].

Current dialysis therapy has improved the prognosis of renal patients dramatically. In fact, in the field of long-term organ substitution, hemo- or peritoneal dialysis has been the most successful form of long term organ substitution therapy, prolonging life expectancy of patients suffering from ESRD with an average of 6.6 years [10]. However, since dialysis therapy is still mainly based on the processes filtration, osmosis and diffusion, the treatment cannot replace the secretory, reabsorptive, and metabolic functions of the kidney. Most kidney disease patients keep experiencing serious medical problems, and their need for chronic intermittent treatment poses a large socio-economic burden. The scientific community is actively investigating possibilities to improve the quality of life for this group of patients. Increased understanding of kidney physiology, together with advances in the technical field of cell culture and tissue engineering, has created opportunities for further improvement of dialysis therapy. In the scope of this thesis, we examined the handling of cationic uremic toxins

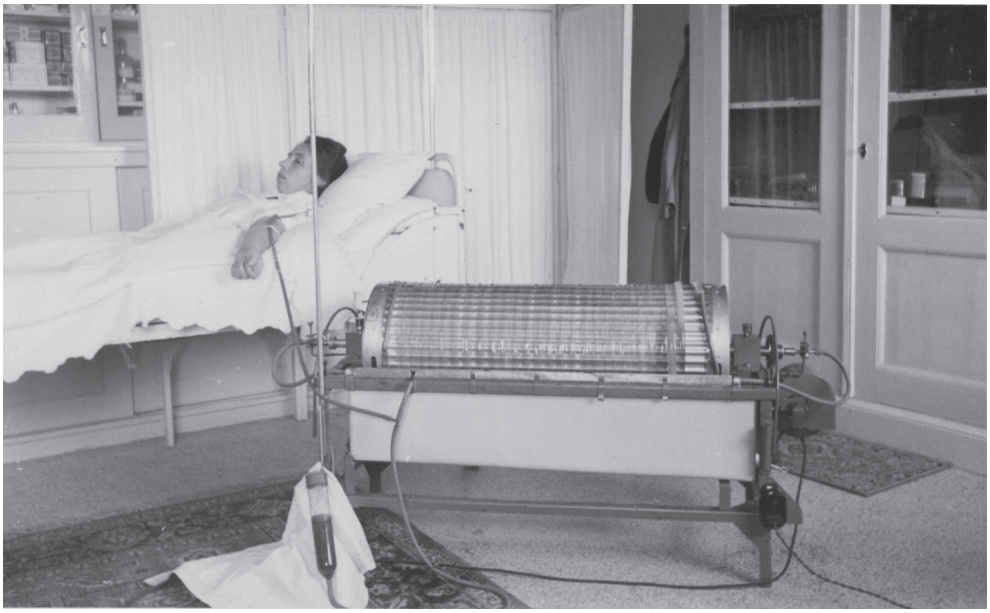

Figure 1.1 The first clinically functional hemodialysis machine for the treatment of CKD.
This first clinically functional hemodialysis machine for the treatment of CKD was presented in the doctoral thesis of Dr. Kolff entitled 'De Kunstmatige Nier' [8]. In this picture nurse Maria ter Welle acts as model patient to show how the device should be installed.

(UTs) by renal tubular epithelial cells. By focusing on the epithelial transport pathways, and regulation involved in the renal removal of these cationic uremic retention solutes, we aim to elucidate the possibilities to utilize living renal epithelial cell membranes for the improvement of hemodialysis therapy.

GENERAL PHYSIOLOGY OF THE KIDNEY

The kidney is an essential organ in the homeostatic regulation of the human body. It facilitates the removal of wastes or foreign substances, produces hormones (such as renin and Epo). The kidney activates Vitamin D, regulates systemic electrolyte balance, pH, and extracellular volume. Approximately 1 million functional units present in the kidney, called nephrons, perform these functions. Nephrons can be subdivided into five sections, made up by the glomerulus, the proximal tubule, the loop of Henle, the distal convoluted tubule and the collecting duct. The three main processes that take place in the nephron are: filtration, reabsorption and secretion (Figure 1.2).

Upon entering the nephron, arterial blood flows through the glomerulus, where filtration occurs under influence of hydrodynamic forces. In a healthy kidney, only substances with a

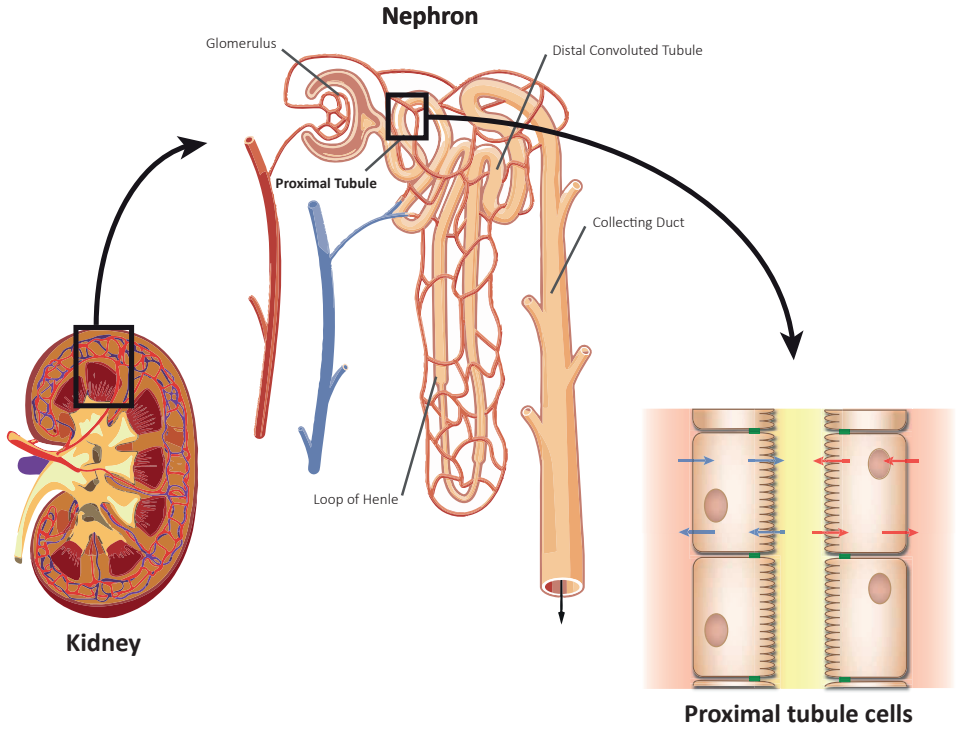

Figure 1.2 Kidney and nephron morphology.
The kidney consists of approximately 1 million nephrons that are localized mainly in the cortex of the kidney. The nephron can be subdivided in five sections, made up by the glomerulus, the proximal tubule, the loop of Henle, the distal convoluted tubule and the collecting duct. We focused especially on the active solute transport taking place in proximal tubule epithelial cells, as proximal tubular secretion processes play an essential role in the removal of xenobiotics such as environmental chemicals, drugs, or endogenous waste products originating from metabolism.

molecular weight up to 7,000 Dalton can freely pass the glomerular filtration barrier. For large molecules, molecular size as well as charge determine the rate of filtration [11]. The filtered fluid therefore consists mainly of water and unbound solutes. Once this fluid passes from the glomerulus into the tubular lumen, it becomes part of the body's external environment. To prevent major loss of fluid, almost all of the filtered water is reabsorbed through channels present in the tubular segments of the nephron. Together with water, the proximal tubule cells reabsorb ions such as Na^+ and Ca^{2+}. Na^+ is actively transported into the extracellular fluid by the Na^+-K^+-ATPase localized on the basolateral membrane. By means of facilitated Na^+-coupled transport the proximal tubule is able to reabsorb a wide range of substances such as PO_4^{3-}, amino acids, glucose and organic metabolites [12]. Furthermore, tubular transcytosis, endocytosis and pinocytosis can mediate the reabsorption of proteins, hormones and enzymes that have passed through the glomerular filtration barrier.

When the solute enters the loop of Henle, urinary concentration takes place [13]. A countercurrent exchange facilitates water reabsorption. In the (thick) ascending limb, active reuptake of Na^+, K^+ and Cl^- causes the fluid to become hyposmotic. The distal convoluted tubule then fine-tunes the electrolyte content by facilitating further sodium chloride reabsorption, potassium secretion, and adjusts Ca^{2+} and Mg^{2+} balance [14]. Subsequently, the collecting system; consisting of the connecting tubule and the collecting duct, makes the final adjustments in urinary concentration [13].

To enhance urinary excretion of substances, the kidney is able to secrete specific molecules. Secretion is very important for the maintenance of body homeostasis, acid-base balance and the removal of xenobiotics or endogenous solutes. Proximal tubular secretion processes play an essential role in the removal of xenobiotics such as environmental chemicals, drugs, or endogenous waste products originating from metabolism.

RENAL SOLUTE CLEARANCE

Renal clearance is a measurement commonly used to analyze renal solute elimination and kidney function. The volume of the renal plasma flow, from which a specific compound is cleared within one minute, is based on the following calculation:

$$CL_{Renal} = \frac{Renal\ excretion\ rate}{C_p}$$

where CL_{Renal} is the renal clearance rate of a solute (mL/min), which depends on the rate of renal solute excretion (g/min), and C_P the plasma concentration of the solute (g/L) [15]. The rate of renal excretion is, in turn, dependent on glomerular filtration, tubular secretion and reabsorption processes, as described in the formula below.

Renal extretion = Filtation + Secretion − Reabsorption

Glomerular filtration

For some substances, elimination will directly reflect the glomerular filtration rate (GFR). One of these compounds is the polysaccharide inulin [16]. Inulin is inert, freely filtered by the glomerulus, and is neither reabsorbed nor secreted by the renal tubules, therefore, CLrenal = GFR, and can describe renal function accurately. However, in clinical settings, physicians prefer the use of the endogenous compound creatinine over inulin [16,17]. As a breakdown product of creatine phosphate, blood creatinine levels are generally stable; therefore this cationic compound does not need to be administered to the patient. While creatinine is filtered freely by the glomerulus, approximately 20% of the creatinine excreted is the result of active

secretion into the urine by organic cation transport proteins. Therefore, creatinine clearance measurements generally overestimate GFR.

Passive filtration by the glomerulus is restricted by two factors. First: the amount of filtered plasma represents approximately 20% of the glomerular blood flow. Meaning that during a single cycle a maximum of 20% of a substance can be excreted by filtration. The second restricting factor is plasma protein binding. Binding of solutes to plasma proteins like albumin facilitates the systemic transport of organic compounds that usually have greater biological activity (toxicity). Protein binding prevents these compounds from passing plasma membranes, thereby influencing their tissue distribution and bioavailability. However, the increased size of protein bound solutes also makes glomerular filtration impossible. As a result, passive clearance by glomerular filtration depends on the free fraction of waste products in the circulation, and the GFR.

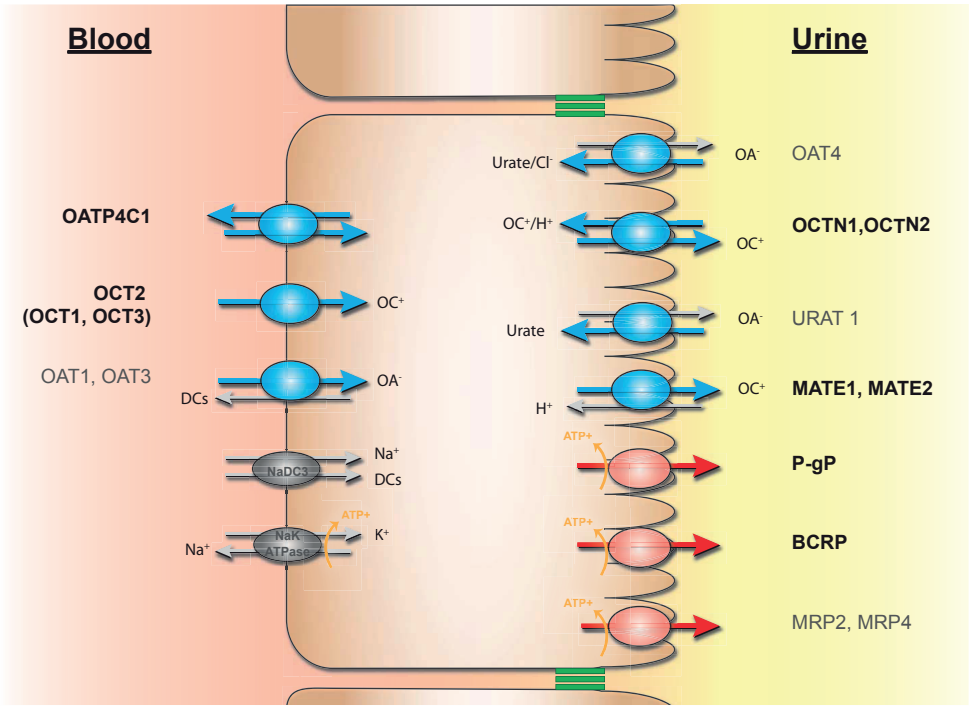

Figure 1.3 Schematic model of the major organic anion (OA^-)/ organic cation (OC^+) transporters in human renal proximal tubular cells.

SLC transporters are depicted in blue and ABC transporters in red. Grey arrows depict the movement of driving ions. In bold transporters are labeled that are currently considered important for the clearance of organic cations. More details are given in the text.

Active proximal tubular organic solute clearance

For solutes that are unable to pass the glomerular filtration barrier due to size or charge, proximal tubular secretion is the primary route of elimination. Organic solutes make up a large part of these waste products. Organic solutes originate from direct ingestion, normal endogenous metabolism or are formed during putrefaction processes occurring in the intestine [18]. The presence of the many dedicated transport pathways in the proximal tubule ensures their rapid excretion (Figure 1.3). The clearance rates of many organic solutes often even exceed the renal plasma flow [19]. Tubular secretion occurs in three subsequent steps. First, the solute diffuses from the peritubular capillary into the interstitium. Then influx transporters carry the solute into the proximal tubule cell across the basolateral membrane. After passing through the cell, the solute is secreted at the apical plasma membrane into the lumen [20].

Proximal tubular organic anion transport

Influx of organic anions is mediated by members of the solute carrier (SLC) family known as organic anion transporter 1 and 3 (OAT1/3; *SLC22A6* and *-A8*) and the bidirectional Organic Anion Transporting Peptide 4C1 (OATP4C1; *SLCO4C1*) [21-23]. As the uptake of negatively charged anions is an energy consuming process, the influx transport of OAT1 and 3 is driven by their exchange for intracellular anions, such as dicarboxylates [24]. The Na^+-dicarboxylate cotransporter (NaDC3; *SLC13A3*), identified in human kidney tissue in 1996, is essential for the maintenance of a cellular dicarboxylate gradient [25]. The driving force for OATP4C1 has as of yet not been identified.

Cellular efflux of organic anions is facilitated by members of the ATP-binding cassette (ABC) transporter family, known as the Multidrug Resistance Proteins 2 and 4 (MRP2/4; *ABCC2* and *-C4*), and Breast Cancer Resistance Protein (BCRP; *ABCG2*), through ATP dependent transport [26,27]. Furthermore, the organic anion transporter 4 (OAT4; *SLC22A11*) and the urate reuptake transporter (URAT1; SLC22A12) mediate the transport of organic anions by their exchange for urate [28,29]. Figure 1.3 depicts a schematic model of the major organic anion and cation transporters in human renal proximal tubular cells

Proximal tubular organic cation transport

At the basolateral membrane of the proximal tubule, the SLC22 family of organic cation transporters (OCTs) mediate organic cation uptake. On the brush border membrane, the SLC47 multidrug and toxin extrusion proteins (MATEs) are expressed. OCTs and MATEs transport a wide variety of structurally unrelated organic cations [30-32]. In the human kidney, OCT2 (*SLC22A2*) is considered one of the most important organic cation influx proteins. Though OCT1 (*SLC22A1*) and OCT3 (*SLC22A3*) are present as well, their renal expression levels are low. In contrast, their transport function in other tissues, such as liver, heart, skeletal muscle, small intestine and lung, is described well [33,34]. In the kidney, the OCT2-mediated basolateral transport of organic cations occurs through facilitated electrogenic diffusion.

OCT2 transport proteins make use of the internal negative membrane potential to allow organic cations to enter into the cell. For proper substrate influx, intracellular concentrations need to remain low, as transport direction is determined by the concentration gradient of the substrate. In order to retain those low intracellular levels of cationic substrates, apical secretion follows rapidly. The apical transporters MATE1 (SLC47A1), MATE2 (SLC47A2), OCTN1 (SLC22A4) and OCTN2 (SLC22A5), work in concert to mediate cation excretion on the apical brush border membrane [35,36]. Apical secretion is mostly driven by an H^+/organic cation antiport process [37]. Also the ABC transporters P-glycoprotein (*ABCB1*; MDR1/P-gp) and the BCRP (*ABCG2)* are involved in the transport of some uncharged and cationic substrates [38-40] (Figure 1.3).

Reabsorption in the proximal tubule

The proximal tubule reabsorbs approximately 67% of the filtered water, and a variety of ions and solutes. In addition, amino acids, glucose and albumin that pass the glomerular filter are reabsorbed. Many active reabsorption processes depend on the proper functioning of the Na^+-K^+-ATPase pump. As this pump keeps the intracellular levels of Na^+ low, the electrochemical gradient can provide Na^+ linked transport of a wide range of substances such as glucose, amino acids, Cl^-, Ca^{2+}, HCO_3^-, and PO_4^{3-}. The recovery of these solutes from the pre-urine to the interstitial space creates an osmotic gradient that promotes the subsequent reabsorption of water through the "leaky" tight junctions or water channels (aquaporins). Filtration of plasma at the glomerulus normally excludes most proteins, but some smaller proteins (i.e. albumin), hormones and enzymes can pass the filtration barrier. Proteins that are too large to be reabsorbed by carriers enter the proximal tubular epithelial cells by receptor-mediated endocytosis at the apical membrane. Both the megalin and cubulin receptors have been identified as key endocytotic receptors that enable selective transport of essential substances from the apical side of the proximal tubule to the basolateral compartment.

RENAL FAILURE, UREMIA AND TRANSPORT OF CATIONIC RETENTION SOLUTES

Renal failure occurs when chronic or acute processes disturb the normal filtration, secretion, and reabsorptive processes of the kidney. CKD is defined by the "Kidney Disease Quality Outcome Initiative" as a reduction of the glomerular filtration rate from the normal rate of $\geq$ 90 mL/min/1.73m^2 to < 60 mL/min/1.73 m^2 for 3 months or more [41]. In the early stages of kidney disease, the loss of nephron function can occur largely unnoticed. In experimental models of CKD, the remaining individual nephrons were found to experience glomerular hyperfiltration, a compensatory mechanism [42,43]. Thus, the adaptive response of the residual nephrons determines for how long normal homeostasis can be preserved. When over

time GFR starts to fall further, the tubular ability for renal urine concentration decreases. Phosphate and potassium excretion diminish, and filtered solutes such as creatinine and urea start to accumulate systemically [44]. When a GFR ≤ 15 mL/min/1.73 m^2 is reached, the osmolarity of the excreted urine starts to resemble that of plasma. In addition, the secretion of organic retention solutes by the proximal tubule fails [45], which leads to severe systemic manifestations of uremia [46].

The uremic syndrome is characterized by symptoms resulting from the accumulation of various organic retention solutes. Symptoms of uremia can include anorexia, nausea, pruritus, itching, anemia and sexual dysfunction. Furthermore, neurological, musculoskeletal, cardiovascular, and immunological problems arise [47]. The development of a chronic inflammatory state together with increased systemic cytokine accumulation is common in renal patients, and uremia is identified as an potential causative factor [48]. The European Uremic Toxin workgroup (EUtox) presently reports over 150 solutes that accumulate during renal failure [49]. Many of these solutes have the potential to act as UTs, because they exert biological or biochemical activities. They are classified into three categories: 1. small, water-soluble, non-protein-bound compounds, 2. larger so-called middle molecules, and 3. protein-bound compounds [50]. Although many candidate toxins are known, it is very difficult to link specific symptoms to solutes. Treatment of end-stage renal disease (ESRD) therefore currently aims to remove as many uremic retention solutes as possible, thereby hoping to alleviate their destructive effects.

Cationic uremic retention solutes

In order to improve the efficiency of the treatment of uremia, studies are executed to identify specific disease causing UTs. The systemic accumulation of several well-studied anionic compounds, such as phenols and indoles, were found to correlate with the development of insulin resistance, epithelial to mesenchymal transition (EMT) and cardiovascular disease [51-54]. Mutsaers *et al.* recently evaluated the effects of accumulating uremic anions, including indoxyl sulphate, indole-3-acetic acid, p-cresyl sulfate, p-cresyl gluceronide, hippuric acid, and kynurenic acid, on renal tubular function [55-59]. The studies showed that these retention solutes can accumulate to high levels in ESRD patients, impede renal transport processes, and affect renal metabolic capacity.

Cationic organic solutes; on the other hand, received less attention. Among these, the guanidino compounds and polyamines, were found to accumulate in plasma of uremic patients [60]. Increased plasma levels of the small water soluble guanidino compounds, such as guanidine, methylguanidine and creatinine (the precursor of methylguanidine), were reported in uremic biologic fluids and tissues [61-63]. Guanidino compounds comprise a large group of solutes, formed during regular metabolism of arginine, originating from proteins and amino acids. The accumulation of these substances in ESRD patients can reach 2-106 times the levels found in healthy individuals (Table 1.1) [50]. The buildup of substances such as guanidine and

Table 1.1 Average normal (CN) and uremic (CU) serum concentrations reported for the selected polyamines, acrolein and guanidino compounds, and their toxic over normal concentration (CU/CN) ratio. As reported in the EUtox Uremic Solutes Database (http://www.uremic-toxins.org/DataBase.html)[49]

Group	Name	State	Av. Reported Normal concentration [µg/L]	Av. reported Uremic concentration [µg/L]	Estimate toxic over normal concentration: (CU/CN) ratio	Solute accumulation can affect:	References
Polyamines	Spermine[a]	protein-binding	-	18 ± 16	n/a	• Erythropoiesis • Erythroid colony formation • Cell growth • Membranes of cytoplasm, mitochondria and nucleus	[77-79,99]
	Spermidine[a]	protein-binding	-	100 ± 45	n/a		
	Cadaverine[a]	protein-binding	-	12 ±11	n/a		
	Putrescine[a]	protein-binding	21 ± 8	77 ± 30	4		
Polyamine metabolite	Acrolein [b]	protein-binding	28 ± 10	76 ± 5	3	• Cell proliferation and apoptosis • Protein function (adducts) • Cellular oxidation status • Membranes of mitochondria and endoplasmic reticulum • Immune response	[80-84,86]
	Acrolein [a]	protein-binding	1700 ± 500	9800 ± 400	6		
Guanidino compounds	Methylguanidine[a]	water-soluble	<7	774 ± 509	106	• Hemolysis • Neuronal function • Cardiovascular system • Immune response	[64-67,69,100,101]
	Guanidine[a]	water-soluble	<12	173 ± 84	15		
	Creatinine [a]	water-soluble	<12	136 ± 46	11		[102,103]

Values are shown as mean (CU); serum concentration in healthy population, and (CN) highest mean uremic serum concentration in uremic patients.
a: Total serum concentration
b: Unbound concentration in serum

methylguanidine affects the immune response, hematopoietic cell functioning and can induce neuronal and cardiovascular adverse events [64-69]. These contributing effects of guanidino compounds to clinical symptoms observed in the uremic syndrome, warrants studying the renal transport processes underlying their clearance.

Other cationic retention solutes that are of interest as possible contributors to the uremic syndrome are the polyamines. This group of protein bound solutes includes cadaverine, putrescine, spermine and spermidine. Polyamines are formed after lysine, arginine and ornithine catabolism. In humans, systemic levels of free polyamines reported are generally below their detection limit, as the polyamines are predominantly localized intracellular [70]. However, under uremic conditions levels increased up to 100 μg/L, depending on the type of polyamine (Table 1.1). In healthy individuals, low levels of polyamines regulate cell growth and development [71,72]. These molecules also play essential roles in for example the regulation of ion channels, free radical scavenging and maintenance of membrane and chromatin stability [73-76]. Though, increased plasma levels of spermine, spermidine, putrescine and cadaverine were found to inhibit erythropoiesis and erythroid colony formation [77,78]. Finally, elevated putrescine levels can inhibit cell growth *in vitro,* and negatively affect cytoplasmic, mitochondrial and nuclear membrane structures [79]. Furthermore, the enzymatic oxidation of polyamines can give rise to toxic metabolites. Sakata *et al.* identified acrolein as a major toxic compound produced from spermine and spermidine by the enzyme "serum amine oxidase" [80]. Acrolein is strongly protein bound. In the healthy situation the total concentration reported was approximately 1.7 ± 0.5 mg/L serum, which included the protein bound fraction. However during renal failure, this level can increase up to 5.8 times (Table 1.1). Already at low doses, acrolein can inhibit cell proliferation and is thought to enhance apoptosis from secondary toxic assaults [81]. Furthermore, acrolein has a deleterious effect of protein function by the formation of adducts with lysine and histidine, gives rise to oxidative stress, mitochondrial disruption, membrane damage, endoplasmic reticulum stress, and can lead to immune dysfunction. [82-84]. Besides the endogenous formation of acrolein during metabolism, exposure to this compound can occur through occupational exposure in the chemical industry, inhalation from (cigarette) smoke and by dietary ingestion [85]. Studies suggest that acrolein production increases during uremia, through accumulation of polyamines resulting from a decrease in their urinary excretion. Increased acrolein levels are found in patients suffering from diabetic nephropathy, chronic glomerulonephritis and nephrosclerosis. [86].

Knowledge on the clearance and effects of cationic uremic retention solutes during uremia is still largely elusive. The active organic cation transport system present within the proximal tubule is thought to be primarily responsible for their removal [87]. Various transporters, such as OCT2 and MATE1/2k, expressed at the basolateral and apical membranes of proximal tubule cells are believed to work in concert to enable the urinary efflux of cationic uremic retention solutes.

Cytokine production and regulatory transport effects of uremic solutes

Next to the intrinsic toxicity of uremic solutes, the development of a chronic inflammatory state is common in uremia. Clinical studies have demonstrated a correlation between increasing IL-6, IL-8 and TNFα cytokine levels and reduced glomerular filtration [88,89]. Increased cytokine production in combination with inefficient clearance of the inflammatory mediators during uremia might cause these effects [48]. During renal failure, both elevated IL-6 and IL-8 levels correlated with increased mortality and poor disease outcome [90]. Furthermore, TNFα is known as an important factor in the development of renal fibrosis, and induces the production of additional inflammatory mediators such as endothelin-1 (ET-1) [91-93]. In healthy subjects, ET-1 functions as a potent peptide regulating the vascular tone, blood flow, and water and salt homeostasis. However, in patients suffering from renal disease, high levels of ET-1 are linked to a further decline in renal function [94-96], and exposure of the proximal tubule to nephrotoxicants has been shown to induce ET-1 release, leading to reduced ATP-driven solute transport [97,98].

CHALLENGES IN THE BIOARTIFICIAL REMOVAL OF UREMIC RETENTION SOLUTES

Due to the shortage of donor kidneys available for transplantation, ESRD is now treated predominantly with dialysis. Unfortunately, current renal replacement therapies cannot provide the active transport functions necessary for the removal of protein bound and highly compartmentalized uremic solutes such as polyamines and guanidino compounds. The inability of current dialysis therapies to take over all of the highly specialized functions of the human kidney is clearly indicated by the survival benefit associated with renal transplantation over dialysis therapy [104]. Renal patients treated with dialysis still suffer from the complications and progression of CKD, associated, amongst others, with the systemic accumulation of retention solutes [105-108].

Utilizing renal proximal tubule epithelial cells in a bioartificial kidney

As a further improvement to existing synthetic dialysis procedures, the field of tissue engineering is exploring how cultured renal cells handle the clearance of UTs. Current research aims to examine the possibilities to utilize the functional properties of renal cells, in terms of their active role in the clearance of uremic retention solutes, in an add-on device to renal hemodialysis therapy (Figure 1.4). A crucial step in the development of such a device is the selection of a suitable cell type. As recently reviewed by Masereeuw *et al.* studies towards proximal tubule specific elimination of (protein-bound) anionic and cationic organic retention solutes, have indicated proximal tubule cells as the preferred cell type for the development of such a bioartificial device [87]. Early studies used animal derived cell-lines to test the feasibility of

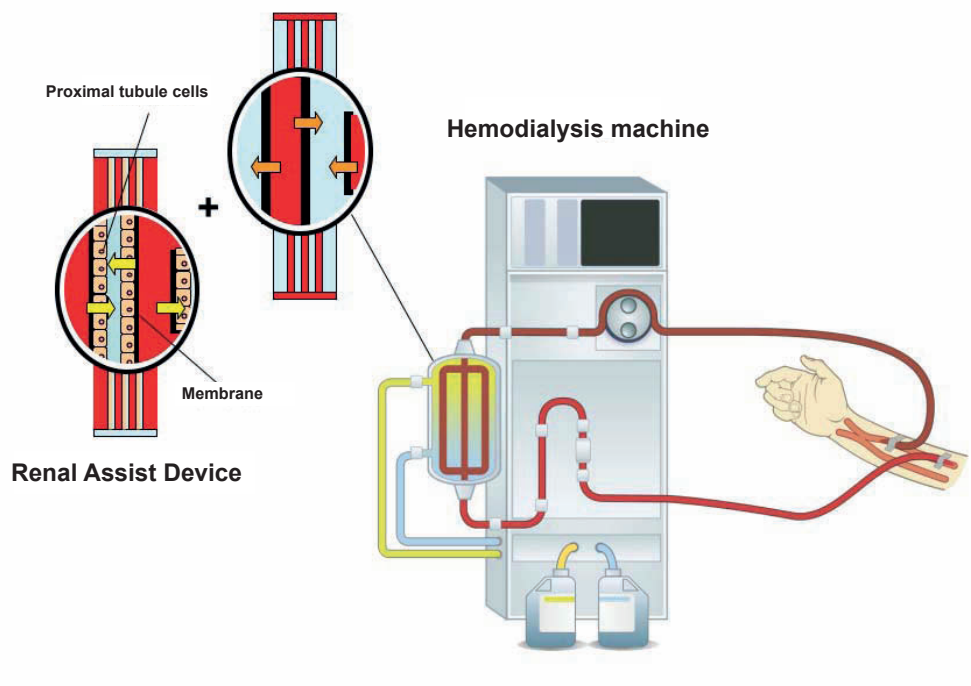

Figure 1.4 Schematic set-up of an add-on device to renal hemodialysis therapy.
This bioartificial kidney, or also called renal assist device (RAD), consists of cultured PTEC monolayers, grown on a semi-permeable hemodialysis membrane. Reprinted with permission from the Dutch Kidney Foundation (©Nierstichting)

a bioartificial kidney. For example, porcine proximal tubular cells (LLC-PK1) were found to retain their reabsorption characteristics and secretion of the cationic compounds tetraethyl ammonium and creatinine by OCT2, under uremic conditions [109-112]. In 1999, Humes *et al.* reported the successful tissue engineering of a porcine cell based bioartificial renal assist device (RAD) [113]. Upscaling of this device even led to the successful treatment of uremic dogs, with regard to ammonia excretion, glutathione reabsorption, and the production of the active form of vitamin D (1,25-$(OH)_2D_3$) -[114].

For clinical development of a bioartificial device, the use of (endogenous) primary human renal proximal tubule cells (PTEC) would be ideal, since these cells still possess most of their proximal tubular characteristics. *In-vitro* experiments have shown functional OAT1/3 and MRP2/4 mediated PAH secretion, MDR1 and BCRP activity, and OCT2 mediated creatinine secretion by tight monolayers of primary hPTEC [115-118]. Promising *in-vivo* results in animal studies with a bioreactor cartridge containing primary human proximal tubule cells, paved the way for the first clinical tests of the bioartificial kidney, or RAD [119,120]. However, the limited number of primary cells that can be isolated from human kidney tissue poses a biological hurdle. To obtain sufficient cells for use in multiple bioartificial devices, *in-vitro* cel-

lular expansion is necessary. But when primary cells are brought into culture, loss of epithelial characteristics and cellular senescence rapidly sets in [121,122], thereby severely restricting the functionality of primary cells *in-vitro*.

The use of immortalized cell lines may be a promising tool for the development of a bioartificial device such as a RAD. In contrast to primary human PTEC, cell lines usually have unlimited growth potential, making large numbers of clonal cells readily available [123]. Some immortalized human renal epithelial cell lines, such as HK-2 or HEK293, are widely used for studying the physiology and pharmacology of the human kidney [124,125]. However, due to their low (endogenous) transporter expression levels, these cells are of limited use for tissue engineering purposes [126,127]. More recently, a conditionally immortalized PTEC line (ciPTEC) was developed from primary PTEC exfoliated in the urine of a healthy volunteer [128]. While exfoliated cells in urine might not be an ideal source for the establishment of a PTEC cell line, its proximal tubular origin has been confirmed by the presence of a brush border membrane, as well as the expression of aminopeptidase N, zona occludens 1 (ZO-1), aquaporin 1 and dipeptidyl peptidase IV [128]. Furthermore, next to mediating albumin and phosphate uptake, the developed ciPTEC cell line was found to functionally express the basolateral cation influx transporter OCT2 as well as the apical efflux transporters p-glycoprotein and MRP4. Additional characterization of this cell line and comparison with ciPTEC from fresh human renal tissue will provide insight into its functional capabilities with regard to the transport of uremic solutes.

Towards development of a living membrane

Next to the availability of functional and well-characterized human PTEC, an important aspect in the development of a bioartificial device is to establish a "living membrane". Such a membrane would consist of tight cellular monolayers, and should be able to maintain the typical polarity and functionality on semi-permeable membranes. Various membrane materials and coatings have been evaluated for both the formation of epithelial tight junctions, whilst retaining biocompatibility [129]. Both Fay-Lamprecht et al. [130,131] and Sato et al. [132] evaluated monolayer growth of renal epithelial cells on a variety of dialysis membranes. These membranes were developed to favor hemocompatibility and prevent cellular adhesion. Therefore, the application of an extracellular matrix (ECM) based coating is essential to promote confluent monolayer formation. While ECM coatings have been reported to improve cell attachment, it appears challenging to maintain functional monolayer integrity [133-137]. Therefore, optimization of the coating process is necessary to prevent the obstruction of membrane pores and to increase the efficiency of the surface coating. Furthermore, a RAD would require cell monolayers to function within a dynamic system, i.e. in presence of fluid flow. Most *in-vitro* knowledge on monolayer formation and transporter functionality currently available has been obtained using static cell culture conditions. The application of flow in cellular systems might influence proximal tubular morphology and functionality. It is, as of

yet, not clear how flow would affect the monolayer and solute transport in ciPTEC. However, several studies reported flow-induced improvement of morphology and solute transport by the tubular epithelium [117,138,139].

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to functionally characterize uremic solute handling by ciPTEC. By evaluating the *in vitro* functional characteristics of cultured ciPTEC monolayers and measuring their endogenous organic cation transport, we strive to gain more insight into crucial cellular aspects, and uremic toxin (UT) elimination pathways important for the development of a bioartificial kidney, or RAD.

Chapter 2 evaluates the effect of cell origin on proximal tubule specific characteristics of two newly developed ciPTEC cell lines from human kidney in comparison to the existing ciPTEC cell line originating from exfoliated renal cells in urine. Next to the comparison of the functionality of key solute transporters, such as OCT2, Pg-P and BCRP, we examine phosphate uptake, ECM forming profiles and morphology of these cell lines. **Chapter 3** examines the OCT-mediated cation uptake in ciPTEC, by measuring cellular cation uptake and inhibition by known substrates. The inhibitory effect uremic guanidino compounds and polyamines on OCT-mediated cation uptake is demonstrated. Furthermore, this chapter shows the cytotoxic potential of these cationic UTs. **Chapter 4** describes the local cytokine and ET-1 production by human proximal tubule epithelial cells exposed to cationic UTs. Both polyamines and guanidino compounds are identified to stimulate the production of IL-6, IL-8, TNFα and ET-1. Furthermore, this chapter evaluates the short- and long-term effects of ET-1 on the regulation of organic cation influx transport in ciPTEC.

The application of cells in a clinical device requires the use of biocompatible materials. Therefore **Chapter 5** focuses on the optimization of ciPTEC monolayer culture on polyethersulfone (PES) based dialysis membranes. We show functional transepithelial transport of creatinine, by ciPTEC monolayers cultured on differently coated PES and polyester cell culture surfaces. For ciPTEC characterization, static culture conditions were used. However, fluid flow plays an important role both in the *in-vivo* situation as well and in a bioartificial renal assist device. Therefore in **Chapter 6** the effect of fluid flow on ciPTEC cation transport is examined, together with the expression levels of various cation transporters, and transepithelial cation transport processes.

The implications of the findings described in this thesis are discussed in **Chapter 7** and finally, both an English and Dutch summary are provided in **Chapter 8**.

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A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue

Jansen J[123]*, Schophuizen CMS[123]*, Wilmer MJ[1], Lahham SHM[5], Mutsaers HAM[123], Wetzels JFM[4], Bank RA[5], van den Heuvel LP[3,6], Hoenderop JGJ[2], Masereeuw R[1†]

* Authors contributed equally

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Departments of [1]Pharmacology and Toxicology, [2]Physiology, [3]Pediatric Nephrology and [4]Nephrology Radboudumc, Nijmegen, The Netherlands.
Department of [5]Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.
Department of [6]Pediatrics, Catholic University Leuven, Leuven, Belgium.

ABSTRACT

Promising renal replacement therapies include the development of a bioartificial kidney using functional human kidney cell models. In this study, human conditionally immortalized proximal tubular epithelial cell (ciPTEC) lines originating from kidney tissue (ciPTEC-T1 and ciPTEC-T2) were compared to ciPTEC previously isolated from urine (ciPTEC-U).

Subclones of all ciPTEC isolates formed tight cell layers on Transwell inserts as determined by transepithelial resistance, inulin diffusion, E-cadherin expression and immunocytochemistry. Extracellular matrix genes collagen I and -IV α1 were highly present in both kidney tissue derived matured cell lines ($p<0.001$) compared to matured ciPTEC-U, whereas matured ciPTEC-U showed a more pronounced fibronectin I and laminin 5 gene expression ($p<0.01$ and $p<0.05$, respectively). Expression of the influx carrier Organic Cation Transporter 2 (OCT2), and the efflux pumps P-glycoprotein (P-gp), Multidrug Resistance Protein 4 (MRP4) and Breast Cancer Resistance Protein (BCRP) were confirmed in the three cell lines using real-time PCR and Western blotting. The activities of OCT2 and P-gp were sensitive to specific inhibition in all models ($p<0.001$). The highest activity of MRP4 and BCRP was demonstrated in ciPTEC-U ($p<0.05$). Finally, active albumin reabsorption was highest in ciPTEC-T2 ($p<0.001$), while Na^+-dependent phosphate reabsorption was most abundant in ciPTEC-U ($p<0.01$).

In conclusion, ciPTEC established from human urine or kidney tissue display comparable functional PTEC specific transporters and physiological characteristics, providing ideal human tools for bioartificial kidney development.

INTRODUCTION

Worldwide, about 2 million people suffering from renal disorders are treated with hemo- or peritoneal dialysis and this number still increases. Main factors contributing to this increase are aging, an increased incidence of diabetes mellitus and hypertension [1-3]. Known limitations of the current dialysis methods as treatment modalities are related to the relatively poor clearance of protein-bound uremic retention solutes [4]. Up to now, a large number of compounds have been classified as uremic retention solutes [5] and their accumulation may have severe clinical implications, such as renal fibrosis, bone disorders, cardiovascular disease and mental disorders [6]. The preferred treatment of patients with end-stage renal disease (ESRD) is transplantation [7], which improves their quality of life and substantially reduces costs associated with extended dialysis [8]. However, tremendous shortages of donor organs as well as complications arising from immunosuppressive treatment and organ rejection after transplantation are profound problems worldwide [9-11].

To replace kidney function, tissue engineering is a promising avenue of research to overcome the limitations of currently available renal replacement therapies [12]. The development of functional and stable human renal epithelial cell models, that are able to actively excrete uremic retention solutes, is a promising step towards a bioartificial kidney device. In the kidney, a heterogeneous cell system is present of which the proximal tubular epithelial cells (PTEC) play an important role in the excretion of endo- and xenobiotics, including uremic retention solutes. The excretory pathway is mediated via a complex interplay involving solute carriers, like OCT2 (SLC22A2), OAT1 (SLC22A6) and OAT3 (SLC22A8) [13], and ATP-binding cassette efflux pumps, such as p-glycoprotein; P-gp (ABCB1), MRP4 (ABCC4) and BCRP (ABCG2) [14]. Besides waste product excretion, reabsorption of filtered solutes, such as phosphate, glucose, urate and albumin, occurs in PTEC [15-18].

In our group, a stable PTEC cell line isolated from human urine was developed (ciPTEC-U) [19]. This cell line demonstrated functional characteristics of important in- and efflux transporters as well as active albumin and sodium-dependent phosphate transport [19], has proven to be valuable in elucidating renal pathological mechanisms [20-23] and in studying renal physiological transport pathways [24,25]. However, as this cell line originates from urine, it could be argued that ciPTEC-U might not reflect the physiological situation as close as cells directly derived from kidney tissue. Cells originating from urine are often thought to be exfoliated from tissue due to apoptosis-induced loss of function [26]. Recently it was shown that overcrowding of epithelial cells due to proliferation and migration, induces the extrusion of living cells to maintain homeostasis in epithelial cell numbers [27]. The aim of this study was to compare ciPTEC-U [19] with newly established human proximal tubular epithelial cell lines from human kidney tissue with respect to important functional properties. Human PTEC were isolated from nephrectomized kidneys followed by subcloning and immortalization techniques. Characterizing the newly established human proximal tubular epithelial

cell lines will allow us to determine whether the sample source influences renal functional properties of cell lines in culture. Insights in these characteristics allows for the identification of the most suitable cell line for further development of a bioartificial kidney device.

MATERIALS AND METHODS

Chemicals and cell culture materials

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. Cell culture plates were purchased from Greiner Bio-One (Monroe, NC) and Transwell inserts were obtained from Corning Costar (cat. no 3460, New York, NY).

Ethics statement

The kidney tissue used for cell line development in this study was obtained from non-transplanted donors, after given informed consent. These organs could not be transplanted due to quality loss of the veins during surgery. No clinical history of renal disorders or any other chronic disease was identified.

Isolation and culture of ciPTEC from kidney tissue

Purification and isolation of renal epithelial cells from kidney tissue was performed as described previously [28]. In short, epithelial cells were isolated by a gradient sieving procedure and subjected to collagenase digestion [29]. The collected primary fraction was transferred to supplemented PTEC culture media: phenol-red free DMEM-HAM's F12 medium (catalogue number 11039, Lonza, Basel, Switzerland) containing 10% (v/v) FCS (Greiner Bio-One, Monroe, NC), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml EGF and 40 pg/ml tri-iodothyronine. Primary cells were immortalized within the first two passages. Immortalization was performed using a combination of hTERT and SV40t as described previously [19,30]. To obtain a homogeneous culture, cells were subcloned using irradiated NIH 3T3 fibroblast as non-dividing feeder cells [30]. After culturing for 2 weeks at 33°C, 5% (v/v) CO_2, single cell clones were visible and picked using cloning discs drained in trypsin-EDTA (MP Biomedicals, Solon, OH). Single cell clones were transferred to a well plate and grown until confluent at 33°C, 5% (v/v) CO_2. Optimal seeding conditions were determined for each obtained ciPTEC line in well plates and Transwell inserts (50 μg/ml collagen IV coating for ciPTEC-U (C6745-1ml)) by testing morphological characteristics and monolayer integrity properties using a cell density range (data not shown). According to the conditions described previously [19], ciPTEC were cultured for 24 h at 33°C 5% (v/v) CO_2 to proliferate and subsequently transferred to 37°C, 5% (v/v) CO_2 for 7 days to mature. Up to at least 40 cell passage numbers were used to investigate proliferation and functional properties following prolonged culturing. Phase contrast images were captured using a Leica DM IL phase contrast microscope.

Immunocytochemistry

To investigate morphology and polarization characteristics of the ciPTEC monolayers, immunocytochemistry was performed using cells cultured on polyester Transwell inserts. Matured ciPTEC were fixed using 2% (w/v) paraformaldehyde in HBSS supplemented with 2% (w/v) sucrose for 5 min and permeabilized in 0.3% (v/v) triton X-100 in HBSS for 10 min. To prevent non-specific binding of antibodies, cells were exposed to block solution containing 2% (w/v) bovine serum albumin fraction V (Roche, Woerden, The Netherlands) and 0.1% (v/v) tween-20 in HBSS for 30 min. Cells were incubated with antibodies diluted in block solution against the tight junction protein zonula occludens 1 (ZO-1, 1:50 dilution, Invitrogen, Carlsbad, CA) for 1 h, followed by incubation with goat-anti-rabbit-Alexa488 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, The Netherlands) for 30 min. Finally, DAPI nuclei staining (300 nM, Life Technologies Europe BV) was performed for 5 min. Protein expression and localization were examined using the Olympus FV1000 Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan) and images were captured using the Olympus software FV10-ASW version 1.7. Next, cell size measurements were performed using Image J software (version 1.40g).

Transepithelial barrier functions

Transepithelial resistance of matured ciPTEC monolayers on Transwell inserts was measured using the Millicell electrical resistance volt-ohm system (Millipore, Billerca, MA). Measurements were performed as described in the manufacturer's protocol. To determine the tightness of the ciPTEC monolayers, inulin-FITC (Sigma-Aldrich) diffusion was measured of matured ciPTEC cultured on Transwell inserts. Both apical and basolateral compartments were washed once with HBSS buffer (Life Technologies Europe BV), prior to 0.1 mg/ml inulin-FITC in HBSS basolateral exposure for 1h at 37°C, 5% (v/v) CO_2. Fluorescence was detected by measuring samples (200 µl) at excitation wavelength 485 nm and emission wavelength 535 nm, using a CytoFluor II Microplate reader (MTX Lab Systems, Vienna, VA).

To investigate monolayer development further, the presence of E-cadherin, a calcium-dependent cell-cell adhesion protein abundantly expressed in PTEC cells [31], was investigated in proliferating and matured cells. After harvesting, cells were fixed and permeabilized using 4% (w/v) PFA and 0.1 % (v/v) saponin in HBSS on ice for 10 min. After centrifuging, cell pellets were resuspended in rat anti-E-cadherin antibody (1:100 in HBSS) and incubated at RT for 30 min. Next, cells were centrifuged again, pellets resuspended in goat anti-rat Alexa 488 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, The Netherlands) and incubated at RT for 30 min. After a final centrifuge step, cells were resuspended in HBSS buffer and measured using a flowcytometer (FACSCalibur BD, software BD CellQuest Pro version 6.0, Becton Dickinson, Franklin Lakes, NJ) gating on live cells (a total of 15,000 cells counted). Separate cell fractions were incubated solely with goat anti-rat Alexa 488 conjugate and signal measured was set as

a negative control. Next to the extracted geometric mean data, representative flowcytometer histograms are shown to illustrate signal intensities (Alexa 488/FL1 signal).

Gene expression of relevant transporters and extracellular matrix proteins in ciPTEC

Total RNA was isolated from proliferating and matured cells using TRIzol (Life Technologies Europe BV) and chloroform extraction according to the manufacturer's protocol. The Omniscript RT kit (Qiagen, Venlo, The Netherlands) was used to synthesize cDNA. The mRNA expression levels of PTEC transporter genes were detected using gene specific primer-probe sets (Table S1, Applied Biosystems, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems). The quantitative PCR reactions were performed using the CFX96 Real Time PCR system (Bio-Rad Laboratories, Veenendaal, The Netherlands) and data were analyzed using the CFX Manager software (Bio-Rad Laboratories). The mRNA expression levels of extracellular matrix (ECM) genes were investigated using primer sets (Table S2) and SybrGreen PCR Master Mix (Qiagen). The quantitative PCR reactions were performed using the ABI 7900HT sequence detection system (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Data of matured cells were normalized to expression levels of the reference gene GAPDH, and were expressed as fold increase compared to matured ciPTEC-U [19]. To compare expression levels from the proliferating towards matured stage per cell line, data of matured cells were expressed as fold increase compared to the corresponding proliferating cells (supplemental figures S1 and S2).

Determination of proximal tubular specific transporter proteins

To detect proteins of interest in proliferating and matured cells, membrane fractions were obtained by ultracentrifugation. Confluent cell layers cultured in T175 flasks were harvested and homogenized in 30 ml buffer containing 18 mM Tris-HCL (pH 7.4), 6 mM EGTA, 0.3 M mannitol and protease inhibitors (100 mM phenylmethane sulphonylfluoride, 5 mg/ml aprotinin, 5 mg/ml leupeptin, and 5 mg/ml pepstatin). The suspension was homogenized using a tight fitting Dounce homogenizer (Kimble Chase LLC, Vineland, NJ) followed by ultracentrifugation (Sorval WX80, Thermo Fisher Scientific, Walhtam, MA) at 100,000 x *g* for 45 min at 4°C. Finally, the membrane pellets were resuspended in 100 µl RIPA buffer containing 1% (w/v) Igepal CA630, 0.5% (w/v) Na-deoxycholate, 0.1% (v/v) SDS (Amersham Biosciences, NJ, USA) and protease inhibitors (0.01% (v/v) phenylmethane sulphonylfluoride, 3% (v/v) aprotinin and 1 mM sodium orthovanadate). The amount of total protein was measured using the Bio-Rad protein detection reagent system (Bio-Rad Laboratories). Protein expression of Organic Cation Transporter 2 (OCT2), P-glycoprotein (P-gp), Multidrug Resistance Protein 4 (MRP4) and Breast Cancer Resistance Protein (BCRP) was investigated by Western blotting using 12%, 6%, 6% and 10% (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), respectively. The iBlot Dry Blotting System (Life Technologies Europe BV)

was used for transferring proteins from the gels onto a nitrocellulose membrane. To prevent non-specific binding of antibodies, membranes were blocked in PBS supplemented with 0.1% (v/v) tween-20 (PBS-T) and 5% (w/v) milk powder (Campina, Woerden, The Netherlands) for 30 min. Subsequently, membranes were washed three times in PBS-T. Next, membranes were incubated for 1.5 h with rabbit anti-OCT2 antibody (1:500, Alpha Diagnostic International, San Antonio, TX), mouse anti-P-gp antibody (1:200, Abcam, Cambridge, UK), rabbit anti-MRP4 antibody (1:100, M49, [32]) or mouse anti-BCRP (1:200, Abcam®, Cambridge, UK). As a loading control mouse anti-β-actin (1:100,000) or rabbit anti-Na, K-ATPase antibody (α-subunit, 1:2,000, C356-M09 [33]) was used. Subsequently, after three washing steps in PBS-T, membranes were exposed to secondary antibodies Alexa fluor® 680 goat anti-rabbit IgG (1:10,000, Life Technologies Europe BV), IRDye 800 goat anti-mouse IgG (1:10,000, Rockland, PA) or IRDye 800 goat anti-rabbit IgG (1:10,000, Rockland, PA) for 1.5 h. Fluorescence was quantified using the Odyssey Infrared Imaging System (version 2.1, LICOR® Biosciences, Lincoln, NE). Data of proliferating and matured cells were normalized to protein expression levels of the loading control and plotted in absolute pixel intensities.

Transport assays of renal in- and efflux proteins

The activity of the renal OCT influx proteins was measured as previously described by Schophuizen *et al.* [25]. In short, harvested matured cell suspensions were exposed to the fluorescent OCT substrate 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) in the presence or absence of 5 mM OCT inhibitor tetrapentylammonium chloride (TPA). Data plotted were corrected for cell numbers.

The activity of the renal efflux protein P-gp in ciPTEC was examined by measuring the amount of calcein accumulation [34]. In short, matured cells were exposed to 1 μM calcein-AM (Life Technologies Europe BV) in the presence or absence of 5 μM P-gp inhibitor PSC833 (Tocris Biosciences, Bristol, UK) for 1 h at 37°C, 5% (v/v) CO_2. Fluorescence in lysed cells was measured and data plotted were corrected for protein concentrations.

To evaluate transport characteristics of the renal efflux transporters BCRP and MRP4 [32,35], ciPTEC were exposed to kynurenic acid, the end product of tryptophan metabolism. Kynurenic acid is a known uremic retention solute and a substrate for both renal efflux transporters [24,36]. To investigate the transport properties of these proteins, cells were cultured in 24 well plates and matured monolayers were gently washed three times using krebs-henseleit buffer supplemented with 10 mM Hepes (pH 7.4, adjusted with Tris-HCl). Subsequently, cells were pre-incubated with supplemented krebs-henseleit buffer in the presence or absence of 5 μM KO143, a known BCRP inhibitor [37], and 5 μM MK 571 a known MRP inhibitor [38] (Alexis Biochemicals, Leiden, The Netherlands) at 37°C, 5% (v/v) CO_2 for 2 h. After pre-incubation, cells were exposed to 10 μM ^{3}H-kynurenic acid (Scopus Research BV, Wageningen, The Netherlands) at 37°C for 2 h. The uptake was terminated by washing the cells 3 times with ice-cold supplemented krebs-henseleit buffer. Cells were lysed using

0.1% (v/v) triton X-100. To each sample 2 ml of scintillation liquid was added and radioactivity was detected using liquid scintillation counting. Counts measured in supplemented krebs-henseleit buffer (blank) were subtracted and data plotted were corrected for protein concentrations.

Albumin and phosphate uptake assays

To investigate albumin uptake mediated by endocytosis, matured ciPTEC were exposed to serum free medium for 4 h at 37°C, 5% (v/v) CO_2. Subsequently, cells were exposed to 50 µg/ml BSA-FITC and incubated at 37°C, 5% (v/v) CO_2 for 30 min. In addition, experiments were performed at 4°C to inhibit endocytosis. After the incubation period, cells were harvested and centrifuged at 1,500 x *g* for 5 min. The cell pellet was resuspended in 4% (w/v) paraformaldehyde in PBS. Finally, intracellular albumin was measured using a flowcytometer (FACSCalibur BD, software BD CellQuest Pro version 6.0, Becton Dickinson, Franklin Lakes, NJ) gating on live cells (15,000 cells counted). Data was analyzed using FlowJo software (version 9.2) and relative net uptake data was plotted next to the actual flowcytometer histograms.

Phosphate uptake was performed in confluent monolayers cultured at 33°C and 37°C, 5% (v/v) CO_2 with $^{32}PO_4$ (Perkin Elmer, Waltham, MA) as described earlier by Malmstrom *et al.* [19,39]. Cells were cultured in 24 well plates and gently washed three times using wash buffer (20 mM Hepes, 5.6 mM $CaCl_2$, 10.8 mM KCl, 2.4 mM $MgCl_2$, 274 mM NaCl) at 37°C. To determine the sodium- dependent uptake of phosphate, experiments were performed in the absence of sodium by replacing NaCl with 274 mM N-methyl-D-glucamine. The uptake buffer was added for 5 min at 37°C consisting of wash buffer, supplemented with 0.22 mM phosphate with 1.0 µCi/mL $^{32}PO_4$ added as tracer. The uptake was terminated by washing the cells five times with ice-cold wash buffer. Cells were lysed using 0.5 ml 0.05 M Na^+-deoxycholate in 0.1 M NaOH. To each sample 2 ml of scintillation liquid was added and radioactivity was detected using liquid scintillation counting. Counts measured in wash buffer (blank) were subtracted and data plotted were corrected for protein concentrations.

Data analysis

All data are expressed as mean ± SEM Statistical analysis was performed using one-way ANOVA analysis followed by Dunnett's Multiple Comparison test or, where appropriate, an unpaired *t* test with GraphPad Prism version 5.02 (La Jolla, CA). A p-value of <0.05 was considered significant.

RESULTS

Successful development of ciPTEC lines from human kidney tissue isolates

To appreciate the functional capacity of ciPTEC-U [19], new PTEC cell lines were generated from human kidney tissue and characterized to compare with ciPTEC-U. PTEC isolates were obtained from three different kidney donors and primary cells were grown successfully. All cell cultures that were immortalized using SV40t and hTERT, were found resistant to both hygromycin B and geneticin (G418) thereby confirming successful transduction. Next, cells were subcloned to obtain homogenous cell lines after which cell proliferation could be maintained for up to at least cell passage 42 without morphological changes. From the original three kidney isolates, different subclones were obtained. Based on mRNA expression levels of PTEC transporters, morphology and monolayer tightness, two out of twenty six kidney subclones were selected (ciPTEC-T1 and -T2) for detailed characterization. The ciPTEC-T1 and -T2 originated from the same donor and were compared in great detail with ciPTEC-U [19]. Figure 2.1a represents a flow chart of the isolation and selection procedure performed in this study. Representative phase contrast images of the selected clones ciPTEC-T1 and -T2 are shown in Figure 2.1b. Transduction of kidney tissue derived cultures with hTERT solely did not result in suitable cell lines as determined by morphology and cell proliferation (data not shown).

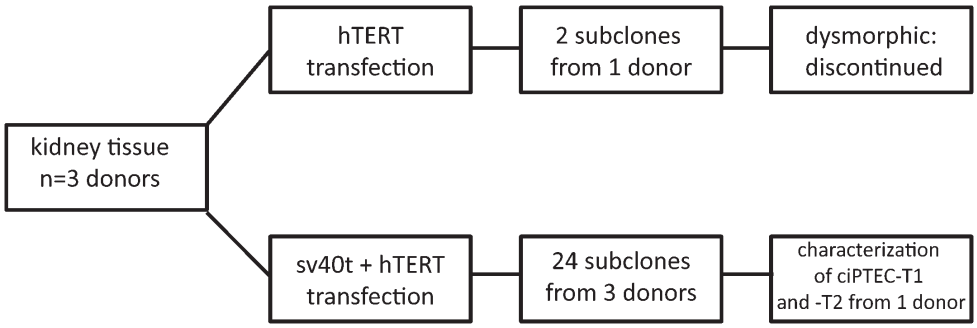

Figure 2.1a Flow chart of clone selection.
Schematic overview of the cell isolation process from kidney tissue upon establishment of clonal cell lines. Cells were isolated from three donors and 26 subclones were selected in a first round. These subclones were characterized morphologically, but also gene expression levels of PTEC specific transporters were investigated. Transduction of kidney tissue derived cultures with hTERT solely did not result in suitable cell lines as determined by morphology and cell proliferation. Finally, two clones from the same donor obtained after combined SV40 and hTERT transductions were chosen for detailed characterization (ciPTEC-T1 and -T2), based on PTEC specific morphological characteristics and highest gene expression levels of PTEC transporters.

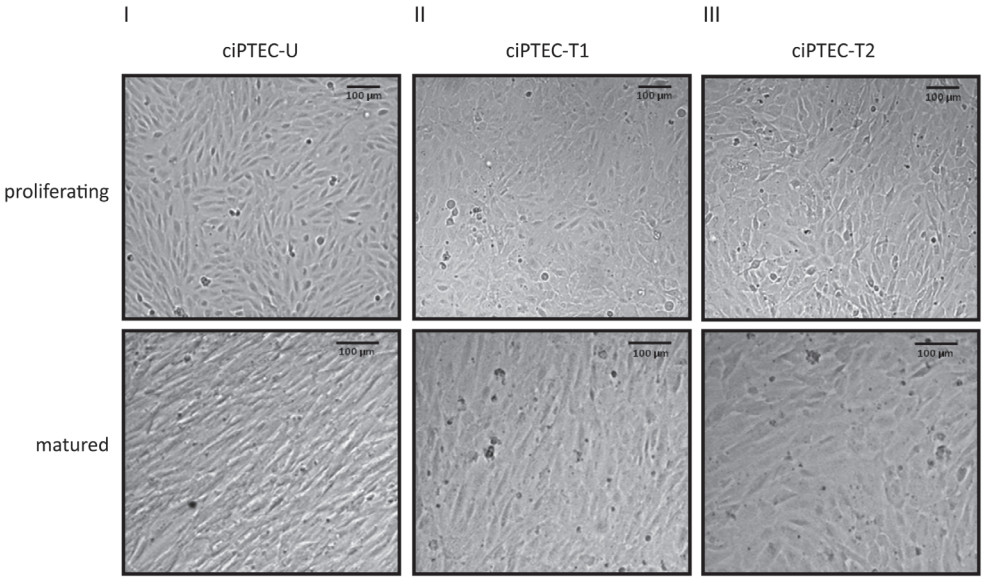

Figure 2.1b Representative phase contrast images of ciPTEC.
Proliferating (33°C, 5% (v/v) CO_2) and fully matured (7 days at 37°C, 5% (v/v) CO_2) ciPTEC-U passage 40 (I), -T1 passage 38 (II), and -T2 passage 42 (III) are shown.

Morphological characteristics and extracellular matrix investigation

Optimal seeding conditions to obtain confluent layers of ciPTEC in well plates and Transwell inserts were determined as indicated in table 2.1. A collagen IV coating of 50 µg/ml stimulated the development of a homogeneous tight layer of ciPTEC-U on Transwell inserts. Interestingly, no coating was necessary for ciPTEC-T1 and -T2 to develop a homogeneous cell monolayer on the inserts (Figure 2.2a - c). In well plates, confluent ciPTEC monolayers were obtained without any additional coatings.

The tight junction protein zonula occludens-1 (ZO-1) in PTEC is a marker for the integrity and polarity of the cell layer. Furthermore, tight junctions contain interacting proteins that regulate differentiation, proliferation and gene expression, indicating an important role in

Table 2.1 Optimal ciPTEC seeding density in well plates and Transwell inserts

Cell line	Well plates seeding density (cells. cm^{-2})	Transwell inserts seeding density (cells. cm^{-2})
ciPTEC-U	4.8×10^4	1.3×10^5
ciPTEC-T1	1.9×10^4	0.60×10^5
ciPTEC-T2	1.9×10^4	0.60×10^5

ciPTEC cultured at indicated densities resulted in tight monolayer formation upon 7 days maturation.

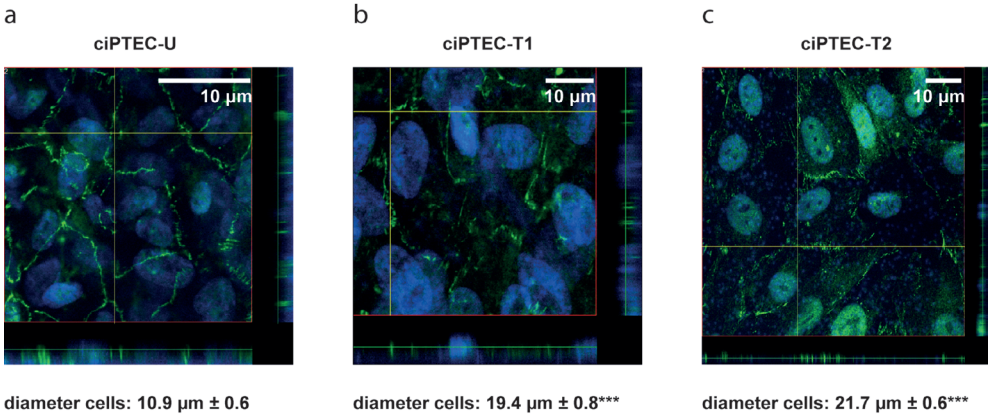

Figure 2.2 Expression of ZO-1 protein in ciPTEC.
Representative confocal images of ZO-1 (green) in x-y and y-z planes are shown (nuclei are stained with DAPI (blue)) in matured (37°C) ciPTEC cultured on Transwell inserts. (a-c) The expression of ZO-1 (green fluorescent) was determined at the cell boundaries and was most abundantly present in ciPTEC-U (a). Furthermore, the average cell diameter was measured, demonstrating a significantly larger cell size of ciPTEC-T1 and -T2 as compared to ciPTEC-U. Note: ciPTEC-U were cultured on collagen IV coated surfaces, whereas ciPTEC-T1 and -T2 were cultured on uncoated Transwell inserts. *** = $p<0.001$, using one-way ANOVA analysis followed by Dunnett's multiple comparison test.

PTEC functionality [40]. Cells cultured on Transwell inserts, were stained against ZO-1 and the protein expression was examined using confocal microscopy. A clear ZO-1 expression was visible for all three cell lines (Figure 2.2a - c). A z-scan depicts the fluorescent signal of tight junction expression at the cell boundaries in each ciPTEC model, but most abundantly in ciPTEC-U, and confirms polarization of the cells. Next to this, we determined cell diameter and observed a larger span for matured ciPTEC-T1 (19.4 µm ± 0.8, $p<0.001$; Figure 2.2b) and -T2 (21.7 µm ± 0.6, $p<0.001$; Figure 2.2c) as compared to matured ciPTEC-U (10.9 µm ± 0.6; Figure 2.2a). Both ciPTEC cell lines isolated from kidney tissue showed a larger span compared to ciPTEC-U when cultured in similar uncoated cell culture flasks, at both temperatures (Figure 2.1b). This indicates that cell size differences were not influenced by a collagen IV coating but may possibly source related.

Cell monolayer tightness was examined further by determination of the paracellular permeability of the cell monolayers using the diffusion marker inulin [41]. CiPTEC cultured on Transwell inserts were basolaterally exposed to FITC-inulin. No differences were observed in inulin flux between the ciPTEC-U versus kidney tissue derived cell lines, as shown in table 2.2. Furthermore, the epithelial barrier integrity was investigated by measuring the transepithelial electric resistance (TEER). Cell lines were cultured on Transwell inserts and the TEER was measured in matured cells (Table 2.2). CiPTEC-T1 demonstrated a higher resistance (140 ±

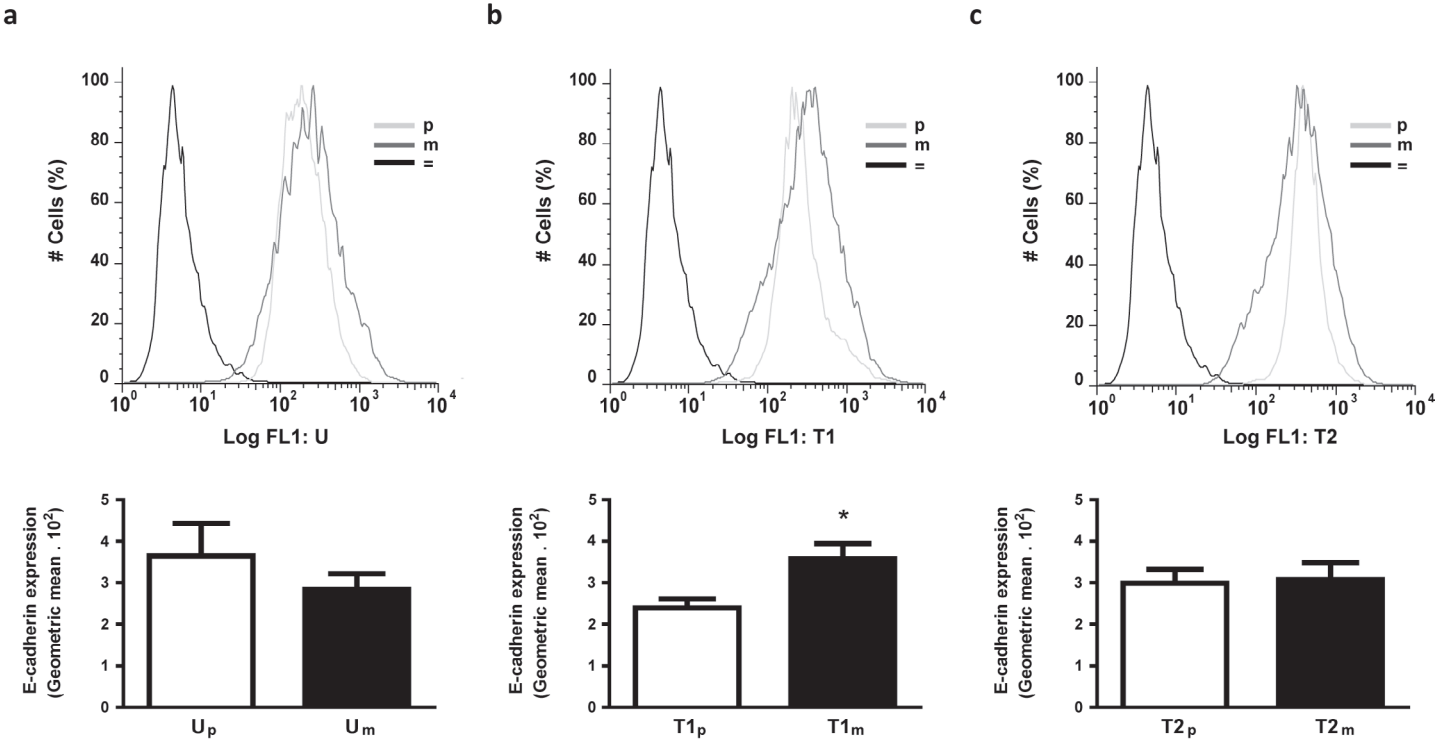

Figure 2.3 E-cadherin expression in proliferating and matured ciPTEC.

(a-c) Representative E-cadherin flowcytometer histograms (FL1/Alexa 488 signal – Log scale) were shown in the upper panel of proliferating (light gray histogram) and matured (dark gray histogram) cells in the tested models. As a negative control (black histogram) ciPTEC were treated according to the protocol but the first antibody incubation step was excluded. (b; lower panel) Upon maturation (black bar), ciPTEC-T1 showed a higher geometric mean as compared to proliferating ciPTEC-T1 (white bar). (a - c; upper panel) Nevertheless, upon maturation all models clearly showed a more heterogeneous population regarding E-cadherin expression compared to proliferating cells. Data are shown as mean ± SEM of three independent experiments. * = $p<0.05$ using an unpaired *t* test.

4 Ω.cm^2, p<0.05) as compared to ciPTEC-U (124 ± 3 Ω.cm^2), whereas no significant differences were observed between ciPTEC-T2 (126 ± 4 Ω.cm^2) and ciPTEC-U

Table 2.2 Transepithelial barrier functions of ciPTEC

Cell line	Inulin diffusion (pmol. min^{-1}. cm^{-2})	TEER (Ω.cm^2)
ciPTEC-U	9.3 ± 0.4	124 ± 3
ciPTEC-T1	8.0 ± 0.7	140 ± 4*
ciPTEC-T2	9.2 ± 0.5	126 ± 4

Monolayer integrity was measured using inulin flux and transepithelial electric resistance determination. * = p<0.05, using one-way ANOVA analysis followed by Dunnett's multiple comparison test. Data presented are means of three independent experiments and expressed as mean ± SEM.

The cell-cell adhesion protein E-cadherin was clearly present in the models tested (Figure 2.3) in proliferating (light gray histogram) as well as in matured (dark gray histogram) ciPTEC compared to the negative control (black histogram), emphasizing the abundant epithelial characteristics of these models. Interestingly, matured ciPTEC showed a more heterogeneous population compared to proliferating ciPTEC. Based on geometric mean data extracted from these histograms, matured ciPTEC-T1 only showed a more abundant prevalence of E-cadherin when compared to proliferating ciPTEC-T1.

To gain more insight in complexes involved in cell development [42,43], the presence of ECM genes was investigated. The mRNA expression levels of collagen I and –IV αI, fibronectin I and laminin 5 (LAMA5; alpha-5 subunit of laminin-10, -11 and -15) were detected in matured ciPTEC-U, -T1 and -T2. Interestingly, significant differences were observed between the cell lines. In matured ciPTEC-T1 and -T2 a higher expression of collagen I - and IV αI (Figure 2.4a and b, p<0.001) was observed compared to matured ciPTEC-U. Whereas fibronectin I and laminin 5 expression was lower in matured kidney tissue derived cell lines (fibronectin I ciPTEC-T1 and -T2: p<0.01, laminin 5 ciPTEC-T2: p<0.05, ciPTEC-T1: not significant) compared to ciPTEC-U. In supplemental figure S1, mRNA expression levels of these genes in proliferating versus matured cells are shown. Matured tissue derived cell lines showed an increased collagen I αI (supplemental figure S1a; p<0.001), fibronectin I (supplemental figure S1c; p<0.001) and laminin 5 (supplemental figure S1d; p<0.05) gene expression, whereas matured ciPTEC-U showed a less pronounced genetic ECM profile. Interestingly, collagen IV αI (supplemental figure S1b) gene expression upon maturation was lower (p<0.001) in each cell line compared to proliferating cells.

Gene and protein expression levels of PTEC specific transporters

The excretion of endo- and xenobiotics in the proximal tubular system is mediated via various important in- and efflux transporters like OCT2, P-gp, MRP4 and BCRP [13,14]. The presence

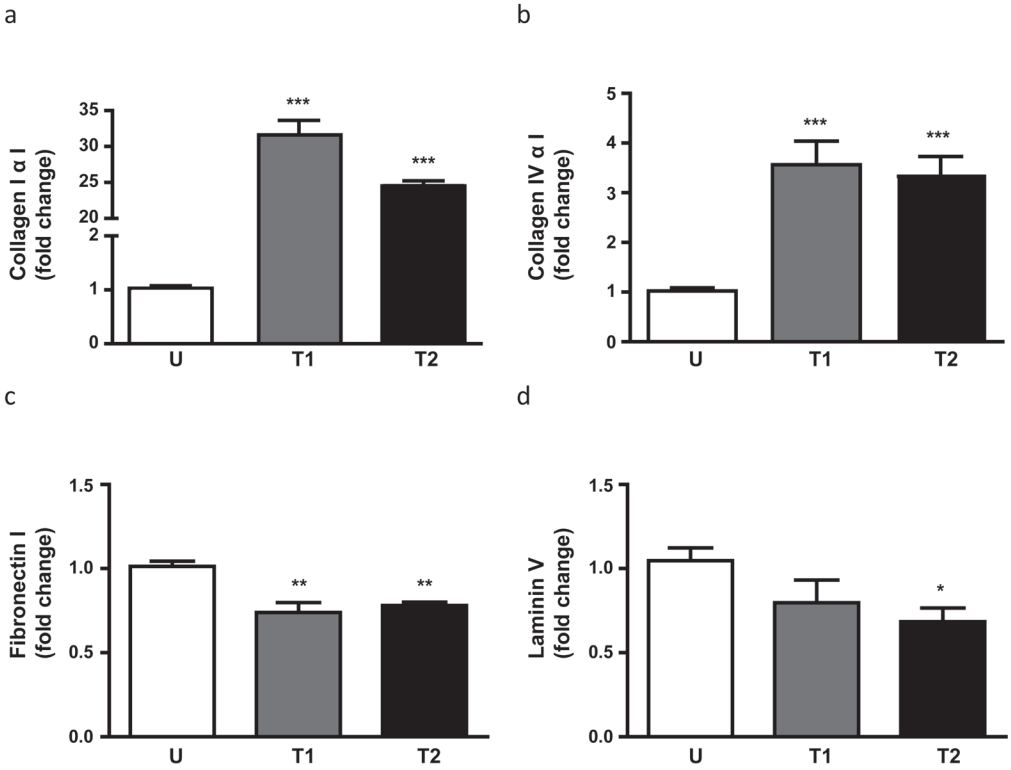

Figure 2.4 Expression of extracellular matrix genes in matured ciPTEC models.
(a and b) Both cell lines derived from kidney tissue showed a higher gene expression of collagen I α1 (a) and –IV α1 (b) compared to ciPTEC-U. (c) In ciPTEC-T1 and -T2, a lower gene expression of fibronectin I was observed compared to ciPTEC-U. (d) In ciPTEC-T2, a lower gene expression of laminin 5 was observed compared to ciPTEC-U. Expression levels were corrected for corresponding GAPDH mRNA levels and data are expressed as fold change and compared to ciPTEC-U. Data are presented as means of three independent experiments ± SEM * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$, using an ANOVA analysis followed by Dunnett's multiple comparison test.

of these PTEC specific transporters was investigated on gene and protein level in matured ciPTEC-U, -T1 and -T2. The mRNA expression levels of most transporters (Figure 2.5) were comparable between the three matured cell lines, except for OCT2 in ciPTEC-T1 ($p<0.05$) and BCRP in ciPTEC-T2 ($p<0.05$) which showed a higher expression as compared to matured ciPTEC-U. In supplemental figure S2, mRNA expression levels of these genes in proliferating versus matured cells are shown. Each matured cell line showed an increased OCT2 gene expression (supplemental figure S2a; ciPTEC-U and -T1 $p<0.05$, ciPTEC-T2 $p<0.001$), whereas P-gp (supplemental figure S2b) and MRP4 (supplemental figure S2c) gene expressions were more abundantly only in matured tissue-derived cell lines (P-gp ciPTEC-T1 $p<0.01$, ciPTEC-

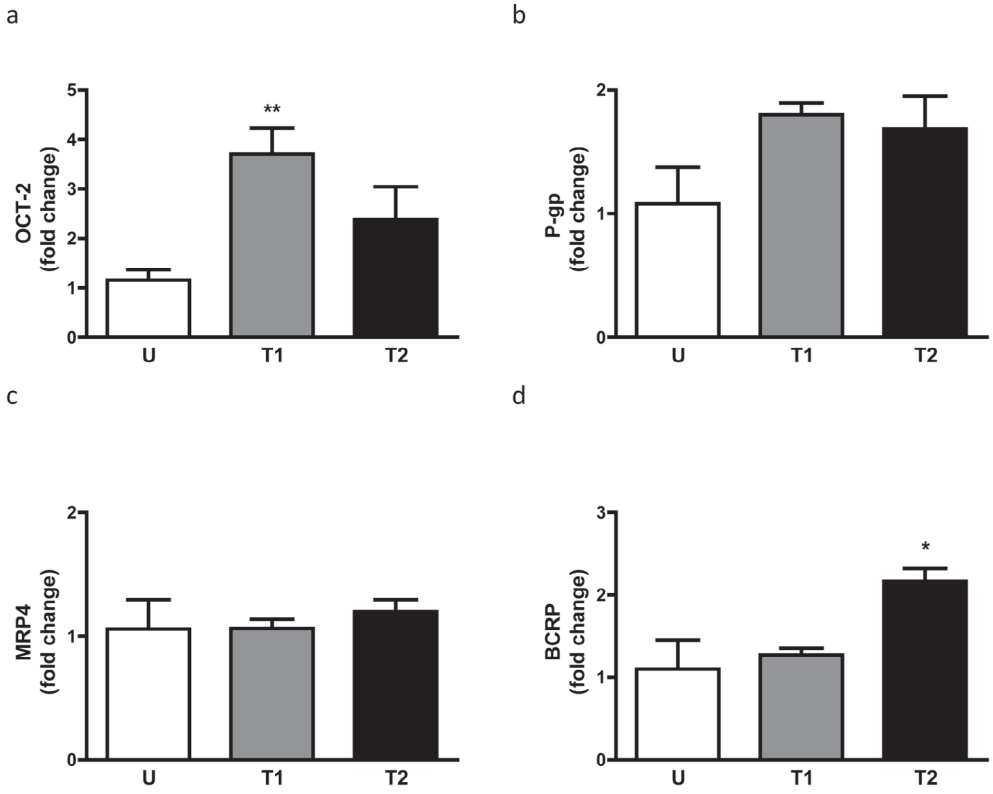

Figure 2.5 Expression of proximal tubular specific transporters in matured ciPTEC models. (a) In ciPTEC-T1 a higher OCT2 gene expression was observed compared to ciPTEC-U. (b and c) No significant differences in P-gp (b) and MRP4 (c) gene expressions were observed between the three cell lines. (d) In ciPTEC-T2 a higher BCRP gene expression wasobserved compared to ciPTEC-U. Expression levels were corrected for corresponding GAPDH mRNA levels and data were expressed as fold change and compared to ciPTEC-U. Data are presented as means of three independent experiments ± SEM * = $p<0.05$, ** = $p<0.01$, using an ANOVA analysis followed by Dunnett's multiple comparison test.

T2 $p<0.05$; MRP4 ciPTEC-T2 $p<0.01$). Although not significant, the gene expression of BCRP (supplemental figure S2d) showed less expression upon maturation in each cell line.

In fully differentiated cells, a non-significant but recurrent trend in abundance of transport protein expression was observed, which points towards a somewhat higher expression of the investigated transporters as compared to the corresponding proliferating cells (Figure 2.6). The expression levels between the different cell lines in their proliferating or matured state was not significantly different.

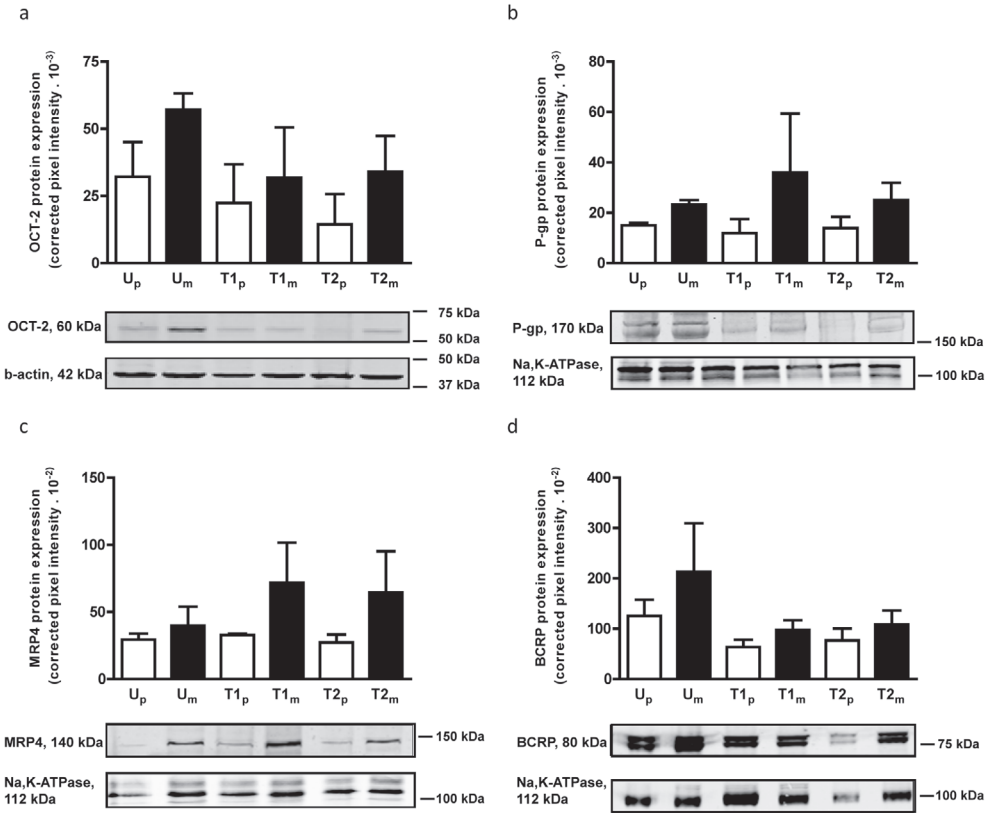

Figure 2.6 Protein expression of OCT2, P-gp, MRP4 and BCRP in membrane fractions of ciPTEC-U, -T1 and -T2.

(a - d) Although not significant, all matured (black bars) ciPTEC showed a slight increased OCT2 (a), P-gp (b), MRP4 (c) and BCRP (d) expression compared to proliferating (white bars) ciPTEC. Signal intensities were corrected for the corresponding reference protein, which was used as a loading control and expression was analyzed semi-quantitative by measuring pixel intensity. Note that due to Western blot conditions Na,K-ATPase appears in multiple bands (b and c). Hence, for semi-quantification the intensity between 100 - 120 kDa was used as a reference. To semi-quantify the OCT2 expression, β-actin was used as a reference protein since the molecular mass of β-actin (42 kDa) is more within the mass range of OCT2 (60kDa) than Na, K-ATPase (112 kDa). The blots presented are representative for protein expression in the ciPTEC models. Data are presented as means of three independent experiments ± SEM and statistical analysis was performed using an ANOVA analysis followed by Dunnett's multiple comparison test.

Functional transport in ciPTEC

To functionally characterize OCTs, we used a recently established assay based on the uptake of the fluorescent marker substrate ASP^+ in ciPTEC suspensions [25] . All three cell lines showed a clear ASP^+ uptake, which was sensitive to inhibition by TPA (p<0.001), in-

dicating active OCTs present in each ciPTEC model (Figure 2.7a). To compare the activity between the cell lines, the net ASP^+ uptake was calculated, which was higher in ciPTEC-U (80 ± 8 RFU/1,000 cells) compared to ciPTEC-T1 (53 ± 2 RFU/1,000 cells; $p<0.05$), whereas no difference was observed between ciPTEC-U and -T2 (net transport 71 ± 5 RFU/1,000 cells).

The functional expression of P-gp was examined by measuring intracellular accumulation of calcein in matured cells, as described earlier [34]. The inhibitor PSC833 was used to obtain accumulated intracellular fluorescent signals due to P-gp inhibition (Figure 2.7b). An increased accumulation was determined in all ciPTEC cell lines ($p<0.001$), indicating

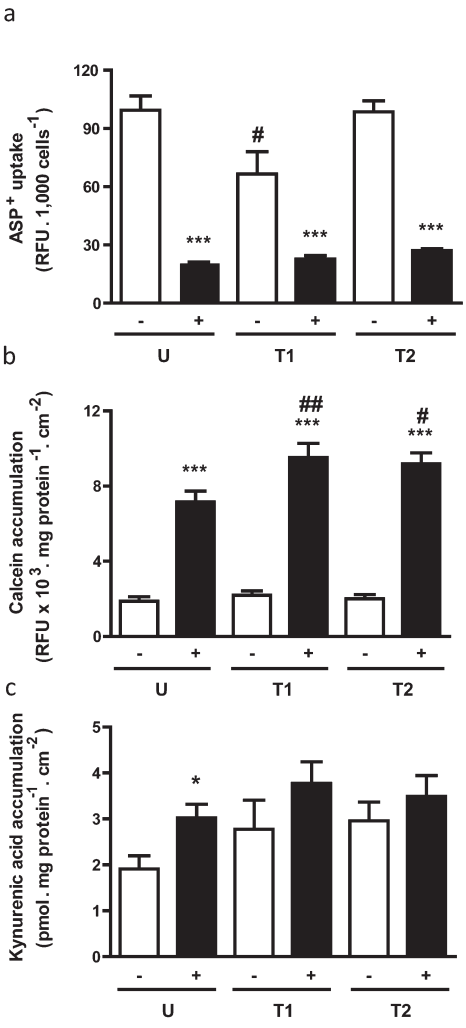

Figure 2.7 Functional in- and efflux proteins in matured ciPTEC.

(a) OCT2 mediated ASP^+ uptake was measured in matured ciPTEC in the presence (black bars) or absence (white bars) of an inhibitor, TPA (5 mM). All ciPTEC models showed ASP^+ uptake which was sensitive to inhibition by TPA, confirming specific OCT activity. In ciPTEC-T1 the net ASP^+ uptake (values without inhibitor subtracted from values in presence of TPA) was lower compared to ciPTEC-U. (b) The P-gp activity in matured ciPTEC was measured in the presence (black bars) or absence (white bars) of a P-gp inhibitor, PSC833. An increased accumulation of calcein was observed in all cell lines in the presence of the P-gp inhibitor. Moreover, in ciPTEC-T1 and –T2 the net calcein accumulation (values without inhibitor subtracted from values in presence of PSC833) was higher as compared to ciPTEC-U. (c) The activity of MRP4 and BCRP in matured ciPTEC was detected in the presence (black bars) or absence (white bars) of the inhibitors, MK571 and KO143. A significant increased accumulation of kynurenic acid was observed in ciPTEC-U in the presence of the inhibitors. Although not significant, a slight increase in accumulation of kynurenic acid was detected in ciPTEC-T1 and -T2. The net kynurenic acid accumulation (values without subtracted from values in presence of both inhibitors) showed no differences between the cell lines. Data are presented as means of three independent experiments ± SEM * = $p<0.05$, *** = $p<0.001$ compared to data in the absence of inhibitors per cell line, using an unpaired t test. # = $p<0.05$, ## = $p<0.01$ compared to net activity in ciPTEC-U, using an ANOVA analysis followed by Dunnett's multiple comparison test.

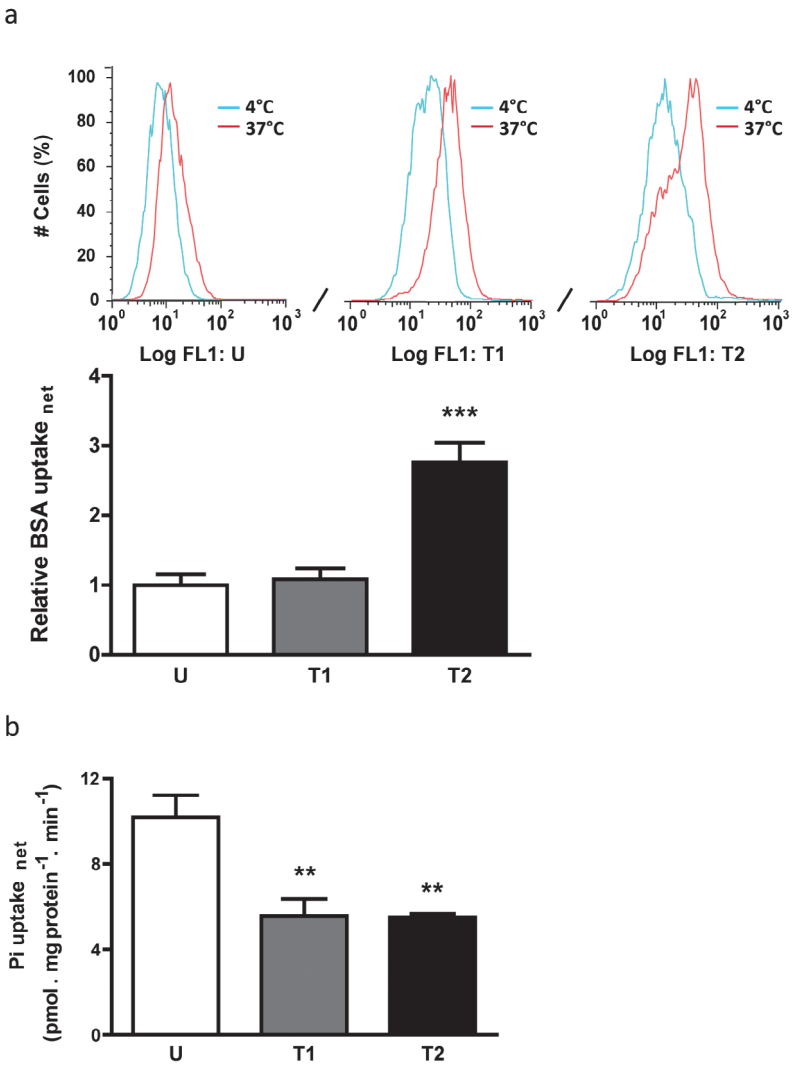

Figure 2.8 Essential reabsorption of albumin and phosphate in PTEC.
(a; upper panel) The albumin reabsorption was detected in matured cells incubated at 4°C (blue histogram) and 37°C (red histogram) during experimental processing (15,000 cells counted). The signal intensities obtained at 37°C clearly showed specific reabsorption in each model. (a; lower panel) The net albumin reabsorption was determined in matured ciPTEC and calculated by subtracting a-specific reabsorption data detected at 4°C from values determined at 37°C. Significantly increased albumin reabsorption was observed in ciPTEC-T2 as compared to ciPTEC-U. (b) The net sodium-dependent phosphate uptake was investigated in matured ciPTEC and calculated by subtracting sodium-independent uptake data from the uptake determined in presence of sodium. A significantly increased sodium-dependent phosphate uptake was detected in ciPTEC-U compared to ciPTEC-T1 and T2. Data are shown as mean ± SEM of three independent experiments. ** = $p < 0.01$, *** = $p < 0.001$, using one-way ANOVA analysis followed by Dunnett's multiple comparison test.

functional P-gp present in each ciPTEC model. To compare the activity between the cell lines, the net calcein fluorescence was determined and a clearly higher calcein accumulation was observed in both cell lines established from kidney tissue (ciPTEC-T1 7.3 ± 0.6 RFU x 10^3. mg protein^{-1}.cm^{-2}; p<0.01 and ciPTEC-T2 7.2 ± 0.4 RFU x 10^3.mg protein^{-1}.cm^{-2}; p<0.05) compared to ciPTEC-U (5.3 ± 0.4 RFU x 10^3.mg protein^{-1}.cm^{-2}).

The functional properties of BCRP and MRP4 were investigated by exposing matured ciPTEC models to kynurenic acid (Figure 2.7c). The compounds MK571 in combination with KO143 were used as MRP4 and BCRP inhibitors, respectively [37,38]. A higher accumulation was observed in ciPTEC-U in the presence of inhibitors (p<0.05). In both tissue derived cell lines, kynurenic acid accumulation was slightly increased in presence of the inhibitors but this effect was not significant. To investigate and compare the BCRP and MRP4 properties between matured ciPTEC models, the net accumulation of kynurenic acid was calculated and compared to ciPTEC-U (1.1 ± 0.4 pmol.mg protein^{-1}.cm^{-2}), but no differences were observed (ciPTEC-T1 0.7 ± 0.6 pmol.mg protein^{-1}.cm^{-2}, ciPTEC-T2 0.9 ± 0.3 pmol.mg protein^{-1}.cm^{-2}).

In addition to uremic toxin (UT) excretion, proximal tubule cells also play an important role in renal reabsorption processes. Wilmer et al. [44] and Gorvin et al. [12] previously reported on the presence of megalin in ciPTEC-U and specific megalin mediated albumin endocytosis was confirmed with the megalin-blocker recombinant receptor-associated protein (RAP). The temperature-sensitive reabsorption of albumin was investigated in the three cell lines (Figure 2.8a; upper panel). The reabsorption was similar in ciPTEC-T1 as compared to ciPTEC-U, whereas ciPTEC-T2 demonstrated a higher uptake of albumin (Figure 2.8a; lower panel; p<0.001).

Another PTEC feature concerns sodium-dependent phosphate re-absorption, mediated via NaPi-IIa and NaPi-IIc and driven by the free energy provided by the electrochemical gradient for Na^+. The transporters are located at the luminal side of proximal tubular cells [16]. The mRNA expression of both phosphate transporters was confirmed in proliferating and matured cells (supplemental figure S3) and uptake in matured ciPTEC-U, -T1 and -T2 was studied (Figure 2.8b). Sodium-dependent phosphate uptake was found to be lower in ciPTEC-T1 and –T2 (p<0.01) as compared to ciPTEC-U. In proliferating cells sodium-dependent phosphate uptake was determined as well (supplemental figure S4), however, the uptake was clearly higher in matured cells as compared to proliferating cells.

DISCUSSION

In this study, human conditionally immortalized proximal tubular epithelial cell lines were successfully developed from kidney tissue. Characterization of the newly established human cell lines and comparison with a cell line isolated from urine revealed that cells from both sources comparably maintain renal physiological properties. A broad range of parameters was

endogenously present in the three cell lines, including the ability to form tight monolayers, ECM deposition and diverse transport activities. On a functional level, the cells isolated from kidney tissue were able to compete with the previously characterized cell line isolated from urine (ciPTEC-U), while collagen I and -IV α1 gene expression was more pronounced in cells derived from renal tissue samples.

To maintain homeostatic cell numbers in the epithelium, live cell extrusion can take place in epithelial cells into the tubular lumen [27]. Although viable cells can be exfoliated in urine, their release might be the result of reduced capability to excrete ECM proteins as demonstrated in this study. The cell lines derived from kidney tissue showed a more pronounced endogenous expression of collagen I and -IV α1 as compared to ciPTEC-U, and monolayer formation of the urine derived cell line was clearly improved by collagen IV coating of culture material. These findings suggest that despite the more abundant gene expression of both fibronectin I and laminin 5 in matured ciPTEC-U, lower levels of essential collagen I and IV α1 expression prevents these cells from developing tight monolayers. In the kidney, the ECM proteins play an important role in intracellular signaling including cell proliferation, survival and migration as well in repair [42,43]. Therefore, it could be argued, that ciPTEC-U has limited properties with respect to ECM and related signaling functions. However, based on the functional data and E-cadherin expression obtained in this study, ciPTEC-U nicely competes with the cells originally derived from kidney tissue. Functionally active OCT2, P-gp, MRP4 and BCRP mediated transport was detected in all models tested, and no superior cell line with respect to the activity of the investigated transport proteins was identified. Furthermore, active albumin reabsorption and sodium-dependent phosphate uptake were measured in ciPTEC-U, ciPTEC-T1 and -T2, with only small variations between the cell lines.

Gene and protein expressions of OCT2, P-gp, MRP4 and BCRP transporters confirmed the endogenous presence of these proteins in ciPTEC-U, -T1 and -T2. The small differences between the three cell models most likely reflect the biovariability. Importantly, the ciPTEC models showed an extensive endogenous expression profile upon maturation, emphasizing the differentiation capacity of these human PTEC lines by immortalization using the temperature sensitive SV40 tsA58 antigen. In the panel of transporters tested, only BCRP was less abundantly present in matured cells as compared to proliferating cells (supplemental figure S2d). This observation can be explained by the role of this efflux pump in kidney regeneration where it has a distinct function during cell development and a less prominent expression upon maturation [45].

Interestingly, although the gene expression levels of the four transporters investigated were lower in matured ciPTEC-U as compared to matured ciPTEC-T1 and -T2, their protein expressions and activities were almost equal in the three models. These observations might reflect differences in post-transcriptional or -translational regulation of the transport proteins. The first step from mRNA to protein can be influenced by epigenetic alterations in signaling molecules, such as Wnt proteins and DNA-binding factors which play a key role in proximal

tubular cell development [46]. The differences in the next step from inactive to a functional and active transport protein can possibly be influenced by an altered activity of kinases and/or phosphatases responsible for phosphorylation and dephosphorylation, respectively [47]. Future research directed towards these pathways should reveal how the four transporters can be modulated in the cell lines.

Robust transport activity was undoubtedly proven for OCTs and P-gp, while the activity of MRP4 and BCRP was less pronounced with the assay used. For the latter transporters the combined substrate kynurenic acid [36] was used in combination with inhibitors for MRP4 and BCRP. A plausible explanation for the limited effect of both inhibitors on kynurenic acid accumulation may be the absence of a specific uptake transporter for kynurenic acid. The uremic metabolite was proven to be an equally potent substrate for the basolaterally expressed organic anion transporters 1 (OAT1) and -3 (OAT3) [48]. OAT1 and OAT3 form important influx transporters in proximal tubular cells and determinants in the excretion of a variety of organic anions, including waste products from normal metabolism and drugs [49,50]. Unfortunately, these transporters are absent on gene, protein and functional levels in the immortalized cell lines isolated from both urine and kidney tissue (data not shown). Although the expression of OATs has been observed in primary proximal tubular cells [51], the levels decrease dramatically during the first days of culturing and are lost after cell passaging (unpublished observations). This phenomenon has already been described in 1990 by J.H. Miller [52] and has, as of yet, not been solved. Stable expression of these OATs in renal cell lines may not only be of importance for studying regenerative nephrology, but may also be of great value for drug development in pharmaceutical industry. Next to the OAT transporters, OATP4C1 is another known anion uptake transporter expressed at the basal membrane of proximal tubule cells [53]. The gene expression was studied in these cell lines and the expression of OATP4C1 was confirmed in matured cells (an average C(t) value of 27.2 ± 0.1 was detected). Assays to demonstrate the functionality of this transporter are in progress. To study the role of transporters in disposition of new pharmaceutical entities and to identify potential drug-drug interactions, a need exists for human models predictive for renal drug handling [44,54,55]. Future research will be directed to further develop and optimize such models.

Next to the functional basolateral uptake and apical efflux transport, active albumin reabsorption and sodium-dependent phosphate uptake are essential processes occurring in human PTEC. Both mechanisms were detected in our ciPTEC models, although differences were observed between the cell lines. These findings underline the heterogeneity with respect to their endocytosis-mediated albumin uptake via megalin, and NaPi-IIa and -IIc mediated sodium-dependent phosphate uptake. As described before, post-transcriptional and -translational differences in these cell lines might explain the observed variability.

Isolating functional renal epithelial cells from human urine and applying conditional immortalization strategies could be a valuable tool in tissue engineering for personalized medicine in patients suffering from renal disorders, e.g. in development of a bioartificial

kidney, or so called renal assist device (RAD)[12]. Indications exist that residual renal function in CKD represents urine produced by tubular secretion rather than glomerular filtration [56]. Residual renal function is a predictor of survival in patients treated with dialysis [57]. Retaining or improving active tubular secretion processes may have profound effects on clinical outcome of CKD patients and the use bioartificial devices may be a good treatment alternative for this patient population [12]. However, the amount and the quality of functional cells in urine originating from these patients might be questionable. In this study, cells from healthy volunteers were transduced using hTERT in combination with the temperature sensitive SV40t gene [19,30]. Although Wieser et al. [58] previously used a single transduction of hTERT only to immortalize primary renal cells, in our laboratory this method did not lead to successful immortalization and hTERT only transductions resulted in dysmorphic cells.

A great advantage of using the combined immortalization strategy is that cells can remain in their proliferating state at 33°C, thereby providing an unlimited cell source. Maturation can be initiated by transferring cells to 37°C, upon which the expression of SV40t in ciPTEC decreases [19] and expression levels of PTEC specific proteins adequately increases (supplemental figure S1 - S3). These findings support their suitability for studying regenerative nephrology. However, the oncogene transductions used require stringent biological safety regulation (e.g. filters absorbing eventually disrupted cells) before implementing in any clinical application, which is the focus of ongoing research and obviously a thorough risk assessment is needed. The use of serum-free culture conditions might be favorable to reduce a possible host immune response. However, culturing the ciPTEC models using serum-free medium for more than 24h induces epithelial-to-mesenchymal transition (EMT) and results in a loss of PTEC-specific characteristics (data not shown). Serum-replacement compounds might be an appropriate alternative, but to this end comprehensive research is required to study the possible effect in these PTEC models prior to implementation.

In conclusion, the human renal lines established from urine and kidney tissue display a comparable variety of functional PTEC specific transporters with maintained reabsorption mechanisms. Interestingly, a different ECM profile was observed in the cell lines isolated from kidney tissue as compared to the cell line isolated from cells exfoliated in urine. The cell models presented here could serve as valuable tools to study proximal tubule physiology and pharmacology. Furthermore, the availability of inexhaustible sources of functional human proximal tubule epithelial cells could allow for further development and up scaling of bioartificial kidney devices.

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SUPPLEMENTARY DATA

Supplementary data is available at http://www.ncbi.nlm.nih.gov/pubmed/24560744

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3

Cationic uremic toxins affect human renal proximal tubule cell functioning through interaction with the organic cation transporter

Schophuizen CMS [1]*, Wilmer MJ [2]*, Jansen J [3], Gustavsson L [4], Hilgendorf C [5], Hoenderop JG [3], van den Heuvel LP [1,7] *, Masereeuw R [2]*

* Authors contributed equally

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Departments of [1]Pediatric Nephrology, [2]Pharmacology and Toxicology and [3]Physiology. Radboudumc, Nijmegen, The Netherlands.
Department of [4]Laboratory Medicine (Malmö), Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden
Department of [7]Pediatrics, Catholic University Leuven, 3000 Leuven, Belgium

ABSTRACT

Several organic cations, such as guanidino compounds and polyamines, have been found to accumulate in plasma of patients with kidney failure due to inadequate renal clearance. Here, we studied the interaction of cationic uremic toxins (UTs) with renal organic cation transport in a conditionally immortalized human proximal tubule epithelial cell line (ciPTEC).

Transporter activity was measured and validated in cell suspensions by studying uptake of the fluorescent substrate 4-(4-(dimethylamino)styryl)-N-methylpyridinium-iodide (ASP^+). Subsequently, the inhibitory potencies of the cationic UTs, cadaverine, putrescine, spermine and spermidine (polyamines), acrolein (polyamine breakdown product), guanidine and methylguanidine (guanidino compounds) were determined.

Concentration-dependent inhibition of ASP^+ uptake by TPA, cimetidine, quinidine and metformin confirmed functional endogenous organic cation transporter 2 (OCT2) expression in ciPTEC. All UTs tested inhibited ASP^+ uptake, of which acrolein required the lowest concentration to provoke a half-maximal inhibition (IC_{50} = 44 ± 2 μM). A Dixon plot was constructed for acrolein using three independent inhibition curves with 10, 20 or 30μM ASP^+, which demonstrated competitive or mixed type of interaction (K_i = 93 ±16 μM). Exposing the cells to a mixture of cationic UTs resulted in a more potent and biphasic inhibitory response curve, indicating complex interactions between the toxins and ASP^+ uptake.

In conclusion, ciPTEC proves a suitable model to study cationic xenobiotic interactions. Inhibition of cellular uptake transport was demonstrated for several UTs, which might indicate a possible role in kidney disease progression during uremia.

INTRODUCTION

In patients suffering from end-stage renal disease (ESRD), kidney function is severely impaired. As a consequence, waste products from normal metabolism are insufficiently cleared from the circulation, resulting in accumulation of uremic retention solutes in the patient [1,2]. Although in ESRD soluble xenobiotics can be cleared by dialysis, the clearance of small lipid soluble and/or protein bound toxins using currently available techniques is insufficient [3]. The accumulation of these UTs contribute to the development of the uremic syndrome, resulting in increased morbidity and mortality [4]. In addition, drug disposition is affected in ESRD patients due to altered pharmacokinetics. Renal failure has been found to affect both renal and non-renal toxin elimination routes [5]. Down regulation of various renal and hepatic drug influx transporters, such as organic anion transporters, p-glycoprotein, multidrug resistance proteins and OCT2, has been observed in ERSD [6,7]. Insights in the mechanism of UT clearance in humans could aid us in the search for new therapeutic possibilities for patients on dialysis.

The human kidney expresses transporters involved in UT clearance. A system involved in renal tubular uptake are the OCT proteins, for which three isoforms (OCT1-3) have been identified [8].The OCT2 (*SLC22A2*) is abundantly expressed in the proximal tubule of human kidney, and is considered essential for the removal of potentially toxic cationic compounds from the circulation, including drugs such as cimetidine (H_2 receptor antagonist), metformin (antidiabetic), and various antibiotics and antihistamines [9,10]. These compounds can cause toxicity via drug-drug interactions due to competitive inhibition caused by the limited transport capacity of OCT2 [11]. Furthermore, OCT2 is a determinant in cisplatin-induced nephrotoxicity due to its role in drug uptake into renal proximal tubular cells [12]. Next to OCT2, the OCT1 (*SLC22A1*) and 3 (*SLC22A3*) isoforms are also involved in cation uptake. While the expression of OCT1 and OCT2 is mainly restricted to the liver and kidney, OCT3 is more widely expressed in various tissues [8]. In normal functioning kidneys, the first step in the urinary excretion of cationic UTs might involve renal tubular cell uptake mediated by OCTs. Indeed, in renal failure several organic cations, such as guanidino compounds and polyamines that are derived from amino acids metabolism, have been found to accumulate in plasma of uremic patients [13].

The guanidino compounds guanidine and methylguanidine are part of a large group of structural metabolites of arginine. Guanidino compounds are small, water soluble cations and not bound to plasma proteins. Despite this, their dialytic removal is insufficient [14]. The accumulation of guanidine and methylguanidine is known to affect hematopoietic cell functioning and can induce neuronal and cardiovascular adverse events [15-18].

The polyamines, cadaverine, putrescine, spermine, spermidine and their breakdown product acrolein, are all protein bound solutes and therefore not freely filtered by the glomerulus. As a result, these solutes are insufficiently cleared through dialysis in ESRD [13]. These

polyamines are formed during cellular lysine, arginine and ornithine catabolism. In humans, serum levels of free polyamines are relatively low as the polyamines are predominantly localized intracellular [19]. Spermine, spermidine, putrescine and cadaverine are reported to inhibit erythropoiesis and erythroid colony formation in a dose-dependent manner [20,21]. High levels of putrescine were reported to inhibit cell growth *in vitro* and to alter cytoplasmic, mitochondrial and nuclear membrane structures [22].

In the present study, we examined the interaction of these classes of cationic uremic retention solutes with OCT-mediated transport in a human conditionally immortalized proximal tubule epithelial cell model (ciPTEC) [23]. To this end, we characterized the interaction of known substrates for OCT2 and studied the inhibitory potency of the guanidino compounds and polyamines on OCT-mediated transport. Furthermore, the cytotoxic potential of the cationic uremic retention solutes was evaluated, as well as the interaction of a cationic UT mixture, mimicking uremic conditions.

MATERIALS AND METHODS

Chemicals

4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) was purchased from Invitrogen (Eugene, Oregon, USA). Tetrapentylammoniumchloride (TPA), metformin, quinidine, cimetidine hydrochloride, spermine, spermidine, cadaverine, putrescine dihydrochloride, acrolein, guanidine hydrochloride, methylguanidine hydrochloride, insulin, transferrin, selenium, tri-iodothyronine, hydrocortisone and epidermal growth factor were purchased from Sigma-Aldrich Co. (Zwijndrecht, the Netherlands).

Cell culture

The immortalized human proximal tubule epithelial cell line ciPTEC was developed as previously described [23]. ciPTEC were cultured in Dulbecco's modified eagle medium DMEM-HAM's F12 (Lonza; Basel, Switzerland) containing 10% v/v fetal calf serum (FCS) (Greiner Bio-One; Alphen a/d Rijn, the Netherlands), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 40 pg/ml tri-iodothyronine. Cells were cultured in the absence of phenol-red and antibiotics. Culture of ciPTEC was done for up to 40 passages, for which proximal tubular characteristics remained unaltered [23]. Cells were seeded at 30% density in tissue culture flasks and cultured for 24h at 33°C 5% v/v CO_2, followed by maturation of the cells at 37°C 5% v/v CO_2 for 7 days prior to experiments.

RNA expression of OCT1, 2 and 3 transporters in ciPTEC

Total RNA was isolated from proliferating and matured cells of interest using TRIzol (Life Technologies Europe BV) and chloroform extraction according to the manufacturers' protocol. The Omniscript RT kit (Qiagen, Venlo, The Netherlands) was used to synthesize cDNA. The mRNA expression levels were detected using gene specific primer-probe sets obtained from Applied biosystems® (OCT1 (SLC22A1) Hs00427552_m1, OCT2 (SLC22A2) hs01010723_m1, OCT3 (SLC22A3) Hs01009568_m1; Applied Biosystems, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems). The quantitative PCR reactions were performed using the CFX96 Real Time PCR system (Bio-Rad Laboratories, Veenendaal, The Netherlands) and data were analyzed using the CFX Manager™ software (Bio-Rad Laboratories). Data were normalized to mRNA expression levels of the reference gene GAPDH. Data were expressed as fold increase compared to proliferating ciPTEC.

Polyacrylamide gel electrophoresis and Western blotting

Cellular homogenates of matured ciPTEC were obtained by scraping cells off a 75-cm^2 tissue culture flask by using a rubber policeman and lysis in 400 µl RIPA buffer containing 1% Igepal CA630, 0.5% Na-deoxycholate, 0.1% SDS, 0.01% phenylmethane sulphonylfluoride, 3% aprotinin and 1 mM Na-orthovanadate. Western blotting was done using reduced 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass., USA). Expression of OCT2 in cell homogenates was analyzed using a rabbit anti-OCT2 (1:500; Alpha Diagnostics, San Antonio, Tex., USA), followed by a goat-anti-rabbit-HRP conjugate (DAKO). As a loading control the household gene rabbit anti-β-Actin was used (1:10000; Alpha Diagnostics, San Antonio, Tex., USA). Human kidney homogenate in RIPA buffer was used as a control.

OCT-mediated ASP^+ transport

OCT activity was measured in freshly harvested matured cell suspensions by addition of the fluorescent OCT substrate ASP^+ in HEPES-TRIS (Hepes-Tris (10 mM), NaCl (132 mM), KCl (4.2 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), D-glucose (5.5 mM), pH 7.4) buffer at 37°C, as described before [24]. The use of this suspension assay allows for high throughput compound screening. We compared the ASP^+ uptake by ciPTEC using this suspension assay at 37°C and 4°C in order to investigate the feasibility of this method as cation influx screening assay (results are given in Online Resource 1). To validate the cation influx measurement further in ciPTEC, cells were pre-incubated for 30 min with compounds known for their OCT2 inhibitory effects; tetrapentylammonium (TPA), cimetidine, metformin, quinidine or verapamil at a concentration range of 5-25,000 µM; prior to co-incubation with ASP^+ (25 µM) for an additional 20 min. Exposure to various UTs; spermine, spermidine, cadaverine, putrescine, acrolein, guanidine and methylguanidine was performed accordingly within a concentration

range of 0.5-5000 μM. The fluorescence intensity was monitored on a Cytofluor 4000 Fluorescence/Bioluminescence Reader (excitation 485 nm, emission 590 nm) for the complete incubation period. ASP^+ uptake was calculated by subtracting the initial fluorescence at t=0 from the endpoint. Uptake was linear for at least up to 60 min (results are given in Online Resource 2). If significant maximal inhibition of ASP^+ uptake was measured, the inhibition constant (K_i) was determined by performing the uptake with ASP^+ at 10, 20 and 30 μM in addition to the respective inhibitor at the concentration ranges indicated.

Additionally, we tested the effect of a combination of UTs on ASP^+ uptake, which represents more closely the situation in uremic patients. To mimic uremic conditions, a mixture of toxins was used comparable to 1000, 100, 10, 1, 0.1 and 0.01 times the uremic plasma concentrations reported in literature (Table 3.2: spermidine 0.67 μM, spermine 0.09 μM, cadaverine 0.21 μM, putrescine 0.88 μM, acrolein 1.42 μM, guanidine 2.18 μM, and methylguanidine 7.66 μM) with which cells were pre-incubated for 30 min followed by ASP^+ uptake measurements as described.

MTT assay

An (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay was performed to measure cellular toxicity of the compounds tested on the ciPTEC [23,25]. In short, cells were seeded onto a 96 wells plate at 30% confluence. After maturation at 37°C for 7 days, the culture medium was removed and the cells were exposed for 30 min to UTs dissolved in culture medium at the concentration range used in the uptake assays. The medium was changed to 5 mg/ml MTT in PBS and the plate was incubated for four hours at 37°C. The intracellular accumulated precipitate was solubilized in 200 μL dimethylsulfoxide. The absorbance was read using a Dynatech MR 580 plate reader using a wavelength of 570 nm from which background was subtracted.

Data analysis

Nonlinear regression analysis was performed using GraphPad Prism version 5.00 for Windows. A sigmoidal or biphasic dose–response model was applied, depending on best fit (determined using the extra sum-of-squares F-test). For the UTs a constraint was applied limiting the top of the curve to a maximum of 100%. To enable detection of partial inhibition, no constraint was applied to the bottom of the curve. The K_i values were calculated by determining the intersections from three separate experiments. Lines of best fit were determined by least square linear regression analysis. Transport inhibition studies were performed in triplicate and repeated at least three times. From the MTT assay, viability was expressed as a percentage of the values obtained from untreated control cells within the same experiment. MTT assays were performed in triplicate and repeated at least three times.

Statistics were performed using GraphPad Prism 5.00 via an unpaired student's t-test or one-way analysis of variance followed by Dunnett's multiple comparison test where appropriate. Differences between groups were considered to be statistically significant when $p<0.05$.

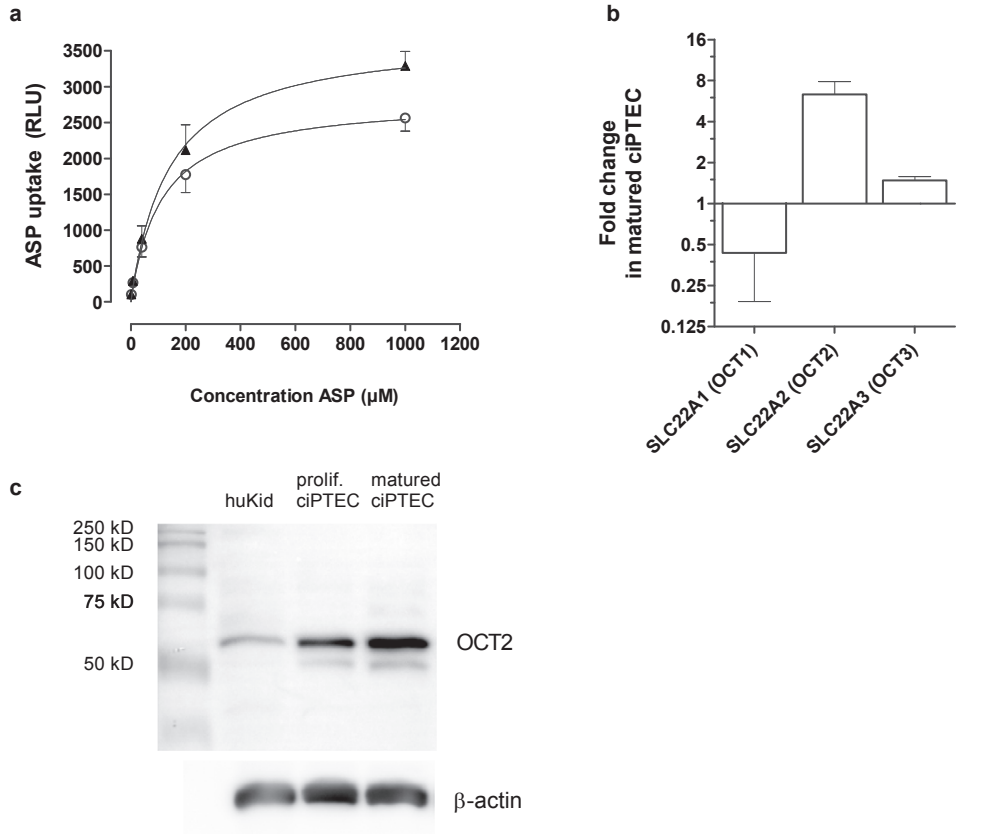


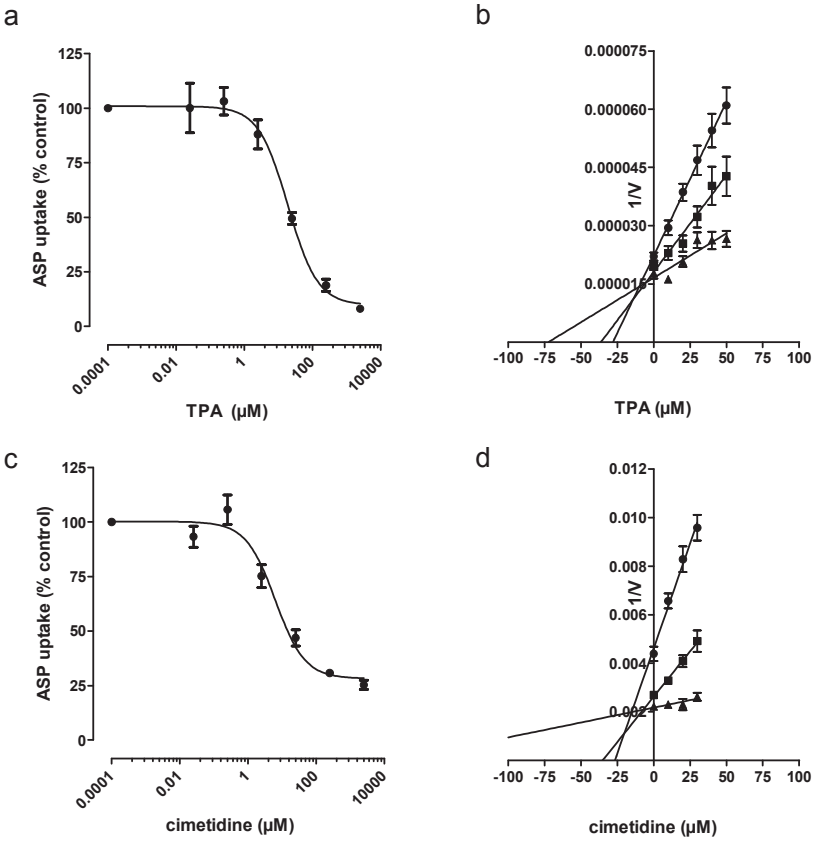
Figure 3.1 Concentration dependent ASP$^+$ uptake, and OCT expression levels in proliferating and matured ciPTEC.

a: Concentration dependent uptake of the fluorescent OCT substrate ASP$^+$ during 20 min by ciPTEC suspensions prepared from proliferating ○ and matured ▲ cells. Data are shown as mean ± SEM of three independent experiments. b: Fold changes in mRNA expression levels of OCT1, 2 and 3 corrected for corresponding GAPDH mRNA levels, expressed as fold increase in matured cells compared to proliferating ciPTEC (proliferating ciPTEC ΔC(t) values OCT1: 13.9 ± 1.1, OCT2: 19.9 ± 0.7, OCT3: 8.4 ± 0.6, and matured ciPTEC ΔC(t) values OCT1: 15.2 ± 0.7, OCT2: 17.7 ± 0.5, OCT3: 7.8 ± 0.5) Data are shown as mean ± SEM of three independent experiments. c: Protein expression of OCT2 examined in lysates of proliferating or matured proximal tubular cells and human kidney tissue using Western blotting. B-actin was used as a loading control. The experiment was performed three times on independent samples, a representative blot is presented.

RESULTS

ASP^+ uptake by ciPTEC is concentration dependent and sensitive to inhibition by TPA, cimetidine, metformin and quinidine

Wilmer *et al.* recently showed functional expression of the OCT2 transporter in the ciPTEC model [23]. To validate further the ciPTEC line as a human kidney cell model suitable to study cation transport, the uptake of the substrate ASP^+ was evaluated using a fluorescence based method [24,26]. Figure 3.1 demonstrates the concentration dependent ASP^+ uptake and OCT expression levels in proliferating and matured ciPTEC. A 6.3 ± 1.4 fold (mean ± SEM) increase in OCT2 mRNA and elevated protein expression levels were observed in matured ciPTEC compared to proliferating cells at 33°C, while changes less than 2-fold were observed for OCT1 and OCT3. The increased expression for OCT2 was further demonstrated on a protein level by Western blotting. Furthermore, ASP^+ uptake increased with 23 ± 5% in fully matured ciPTEC, with an apparent K_m of 139 ± 37 μM for matured ciPTEC versus an



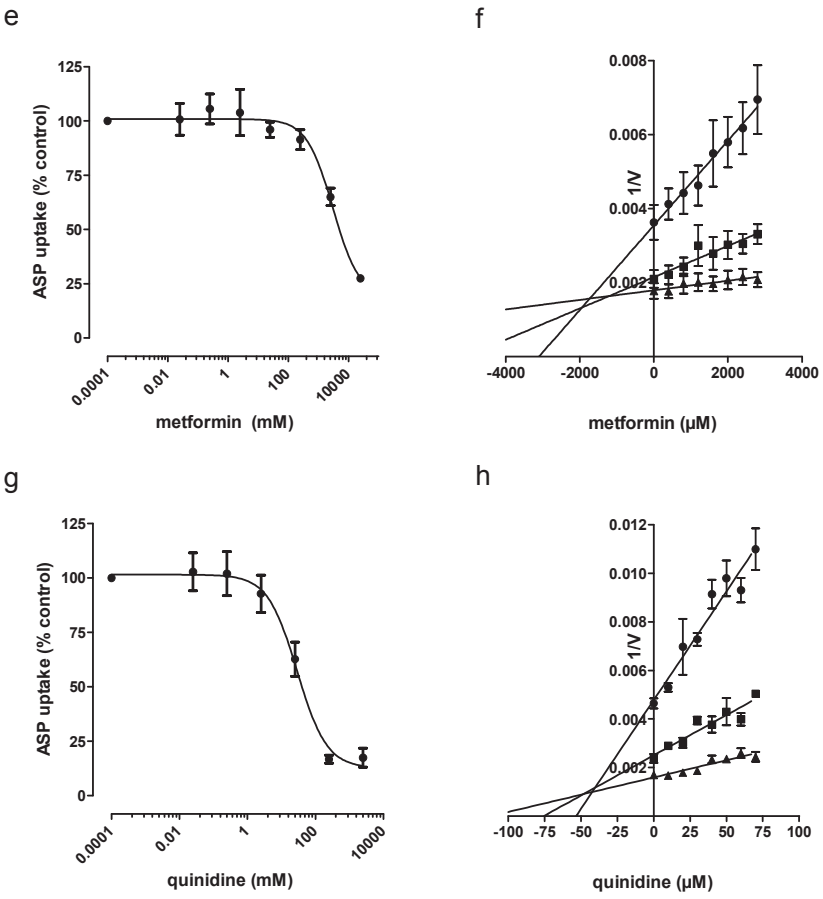


Figure 3.2 Known OCT2 substrates inhibit ASP$^+$ uptake by ciPTEC cells.
Concentration-inhibition curves of ASP$^+$ uptake by TPA (a), cimetidine (c), metformin (e) and quinidine (g). The experiment was performed three times in triplicate. Values are given as mean ± SEM described as percentage of ASP$^+$ uptake in the absence of the inhibitor. Presented IC_{50} curves are an average of three independent experiments. Nonlinear regression was calculated using the GraphPad Prism sigmoidal dose–response model. Corresponding Dixon plots of the reciprocal of OCT2 activity (1/V) versus a concentration range of the known inhibitors TPA (b), cimetidine (d), metformin (f) and quinidine (h). Measurements were performed using ASP$^+$ concentrations of 30(▲), 20(■) and 10(●) µM. Lines of best fit were determined by least square linear regression analysis. Dixon plot experiments were performed three times in triplicate, representative plots are presented.

apparent K_m of 110 ± 28 µM in proliferating ciPTEC. Taken together, these findings suggest ASP uptake in matured ciPTEC is mainly influenced by OCT2.

The ciPTEC line was validated further by examining the effects of known substrates and/or inhibitors of the transporter, namely, TPA, cimetidine, metformin, quinidine, and verapamil

(not shown) on ASP^+ uptake. The results show that the drugs tested effectively inhibited the uptake of ASP^+ by the ciPTEC line in a dose-dependent manner (Figure 3.2a, c, e and g). The calculated half maximal inhibitory concentration values (IC_{50}) are given in Table 3.1. To investigate the mode of interaction, Dixon plots were constructed from the resulting inhibition curves (Figure 3.2b, d, f and h), which were analyzed by linear regression. These plots were consistent with a competitive or mixed-type inhibition for all compounds tested; the K_i values calculated are also presented in Table 3.1. Additional ASP^+ uptake experiments without pre-incubation of the substrates and/or inhibitors of OCT2 did not alter the observed inhibition curves or IC_{50} values (data not shown).

Table 3.1: Apparent IC_{50} and K_i values for the inhibition of ASP^+ uptake by known OCT inhibitors

Known inhibitors	IC_{50} ± SEM (μM)	K_i ± SEM (μM)
TPA	16 ± 2	13 ± 2
Metformin	3954 ± 1245	4271 ± 521
Cimetidine	8 ± 2	36 ± 4
Quinidine	30 ± 5	40 ± 5
Verapamil	10 ± 4	n.d.

n.d. not determined.

UTs inhibit OCT-mediated transport

Cation transporters in the kidney are not only associated with exogenous xenobiotic transport (e.g. drugs), but could determine metabolic waste secretion as well. Therefore, seven UTs were selected to study their effect on OCT-mediated transport in ciPTEC. Their chemical structures and corresponding electrical charges at pH 7.4 are presented in Figure 3.3. The selection was based on the fact that these cationic compounds are protein bound or compartmentalized in patients with renal failure, and accumulate systemically due to insufficient clearance with regular dialysis therapy [27].

The effect of the different toxins on ASP^+ uptake is presented in Figure 3.4. The UTs tested inhibited ASP^+ uptake dose-dependently. The IC_{50} value ± SEM for acrolein was 44 ± 2 μM. The inhibition data of the other compounds did not allow for calculation of accurate IC_{50} values, since the lower plateau of inhibition was not reached. The well-characterized anionic UT, indoxyl sulphate [28], was used to confirm the specificity of the interaction. As expected, this toxin did not affect ASP^+ uptake in ciPTEC (Figure 3.4h). Spermine, spermidine and cadaverine (Figure 3.4a, b and d, respectively), inhibited uptake significantly only at the highest concentration tested (5 mM). Of all UTs tested, acrolein, the end product of both spermine and spermidine metabolism, was the most potent (Figure 3.4g). In contrast, another polyamine, putrescine, was the least potent cationic UT (Figure 3.4c). Furthermore, both

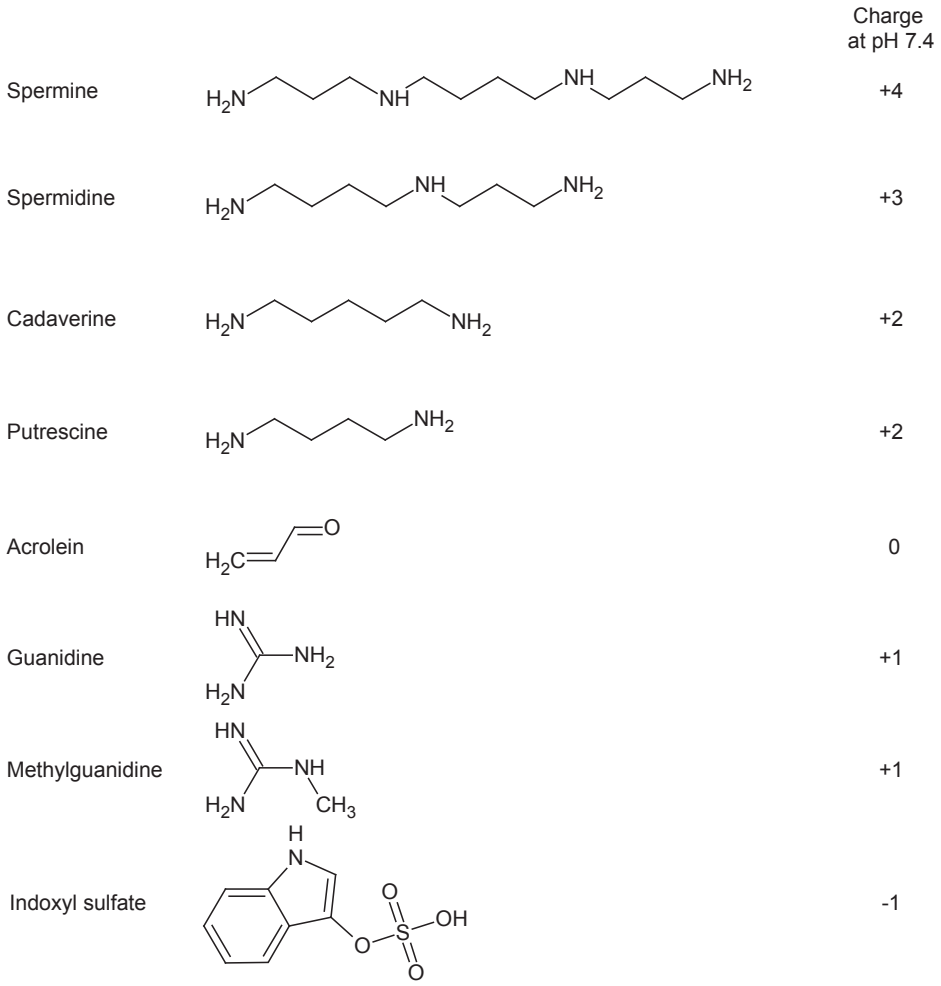


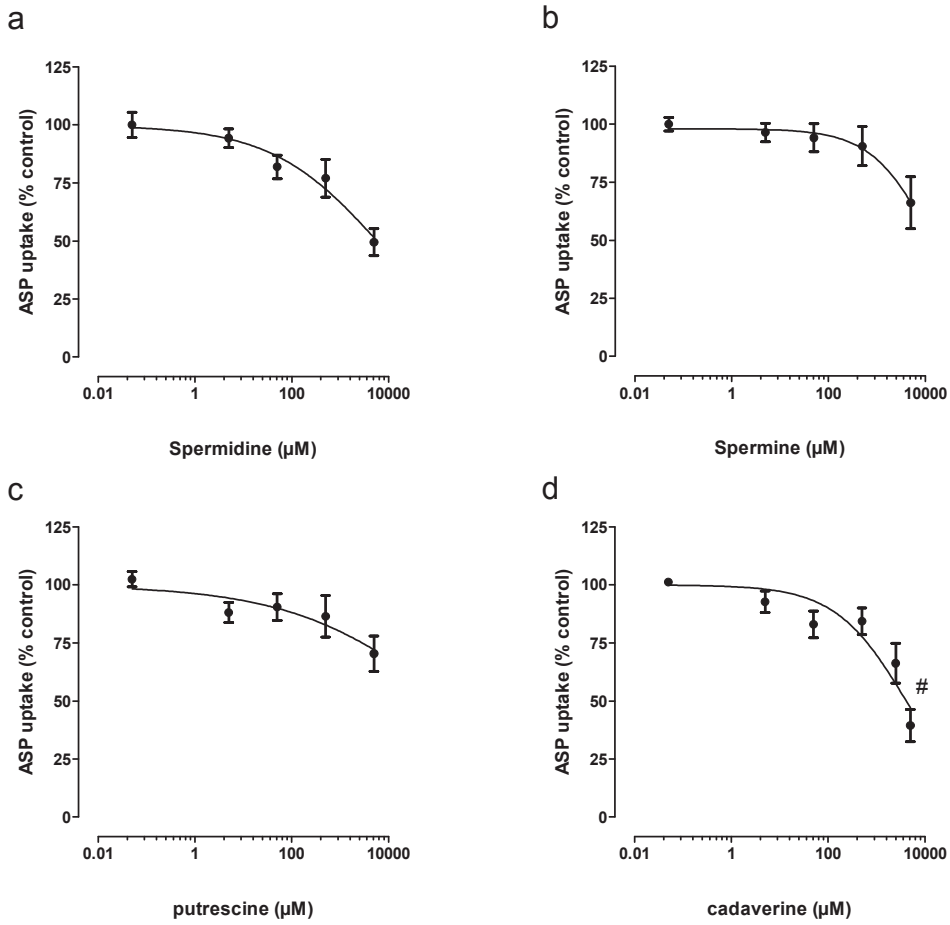
Figure 3.3 Chemical structures and charges of the UTs studied.

guanidine and methylguanidine inhibited ASP^+ transport significantly, of which guanidine showed a higher potency (Figure 3.4e-f, respectively).

For acrolein, the mode of interaction of this toxin with the transporter was studied in more detail. To this end, inhibition was determined using three different ASP^+ concentrations and a Dixon plot was constructed from the individual inhibition curves, which was analyzed by linear regression (Figure 3.5). The Dixon plot is based on the acrolein concentrations within the linear part of the inhibition curve (200, 150, 100, 50, 25 and 0 μM). In agreement with the known OCT2 inhibitors and substrates, inhibition of ASP^+ uptake by acrolein was consistent with a competitive or mixed type of interaction, and a K_i value of 93 ± 16 μM was calculated.

High concentrations of cadaverine, guanidine and acrolein can affect cellular viability

The cytotoxic potential of the UTs was investigated by measuring ciPTEC viability after a 30 min exposure period, corresponding to the exposure time during the OCT2 uptake assay. Exposure to spermine, spermidine, putrescine and methylguanidine did not result in reduced cell viability. For the other UTs a reduction in cell viability was observed only at a concentration of 5 mM (Represented by # in Figure 3.4, panels d, e and g). For cadaverine a reduction in viability of 13 ± 4% was observed when exposed to 5 mM, guanidine resulted in 14 ± 11% reduction in viability and for acrolein viability was reduced with 21 ± 10%. It should be noted that no effect on viability was observed at concentrations below 5 mM.



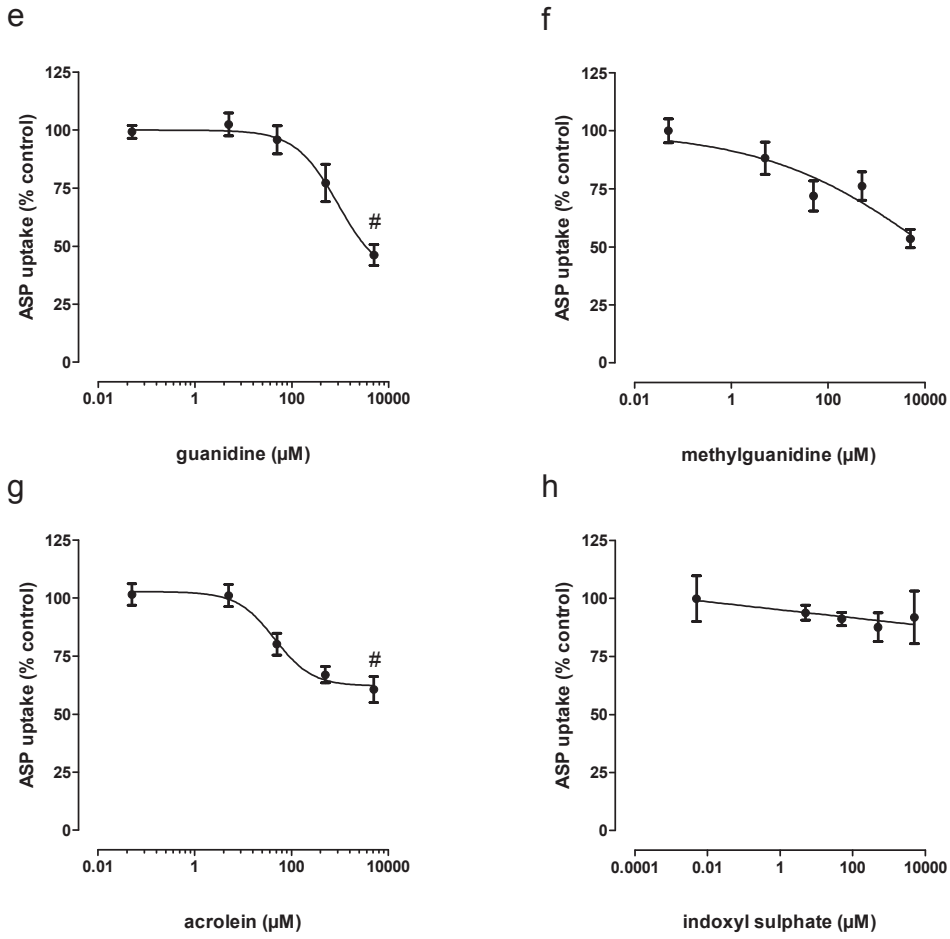


Figure 3.4 Cationic UTs inhibit ASP^+ uptake by ciPTEC cells.
Concentration-inhibition curves of ASP^+ uptake by the polyamines; spermidine (a), spermine (b), putrescine (c), cadaverine (d), and the guanidine compounds; guanidine (e) and methylguanidine (f). In (g) the ASP^+ uptake is shown in the presence of acrolein, a metabolite of spermine and spermidine. In (h) indoxyl sulphate, an anionic UT, was used as a negative control. The experiment was performed three times in triplicate. Values are described as percentage of ASP^+ uptake in the absence of the inhibitor, and given as mean ± SEM. Nonlinear regression was calculated using the GraphPad Prism sigmoidal dose–response model. For acrolein the IC50 ± SEM value was estimated at 44 ± 2 μM. The symbol # indicates the detection of a significant decrease in viability for these concentrations, as determined by the MTT assay. For 5 mM cadaverine, guanidine and acrolein a residual cell viability of 87 ± 4%, 86 ± 11% and 79 ± 10% was observed, respectively.

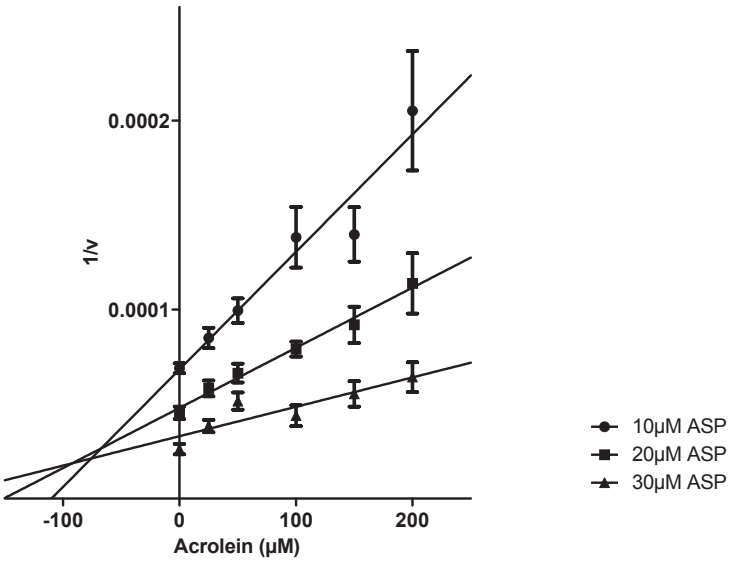


Figure 3.5 Dixon plot of the reciprocal of OCT2 activity (1/V) versus a concentration range of acrolein.

Measurements were done using ASP^+ concentrations of 10, 20 and 30 μM. The experiment was performed three times in triplicate. Lines of best fit were determined by least square linear regression analysis; Ki: 93 ± 16.

A uremic mixture of toxins inhibits OCT-mediated transport

To determine the relevance of the observed inhibitory effects of UTs on ASP^+-mediated transport, we determined the transport function in ciPTEC incubated with a mix of toxins, mimicking more closely the situation in patients with renal dysfunction (Figure 3.6). The polyamine, acrolein and guanidine mixture was prepared at 1000, 100, 10, 1, 0.1 and 0.01 times the free uremic plasma concentrations as reported in literature (Table 3.2). No inhibition of ASP^+ uptake was observed after exposing the cells to a mixture of the polyamines (spermidine, spermine, cadaverine and putrescine; Figure 3.6a). When acrolein was added to the mixture, a large inhibition of ASP uptake was observed only at 1000 fold the uremic concentration (Figure 3.6b). Exposure of ciPTEC to a mixture of only the guanidino compounds (methylguanidine and guanidine) induced a dose dependent inhibition of ASP^+ uptake (Figure 3.6c). The observed transport inhibition at 1x the uremic concentration was 19% ± 6. With acrolein added to this mixture, inhibition did not increase any further (Figure 3.6d).

Interestingly, after exposing the ciPTEC to a mixture of all examined cationic UTs, a biphasic inhibition curve was observed (Figure 3.6f). The estimated IC_{50} values of the first and second inhibitory phase were 0.3 ± 0.3 and 122 ± 50 fold the uremic concentration, respectively. The observed inhibition of ASP^+ uptake at a 1x the uremic concentration was 18% ± 11, which

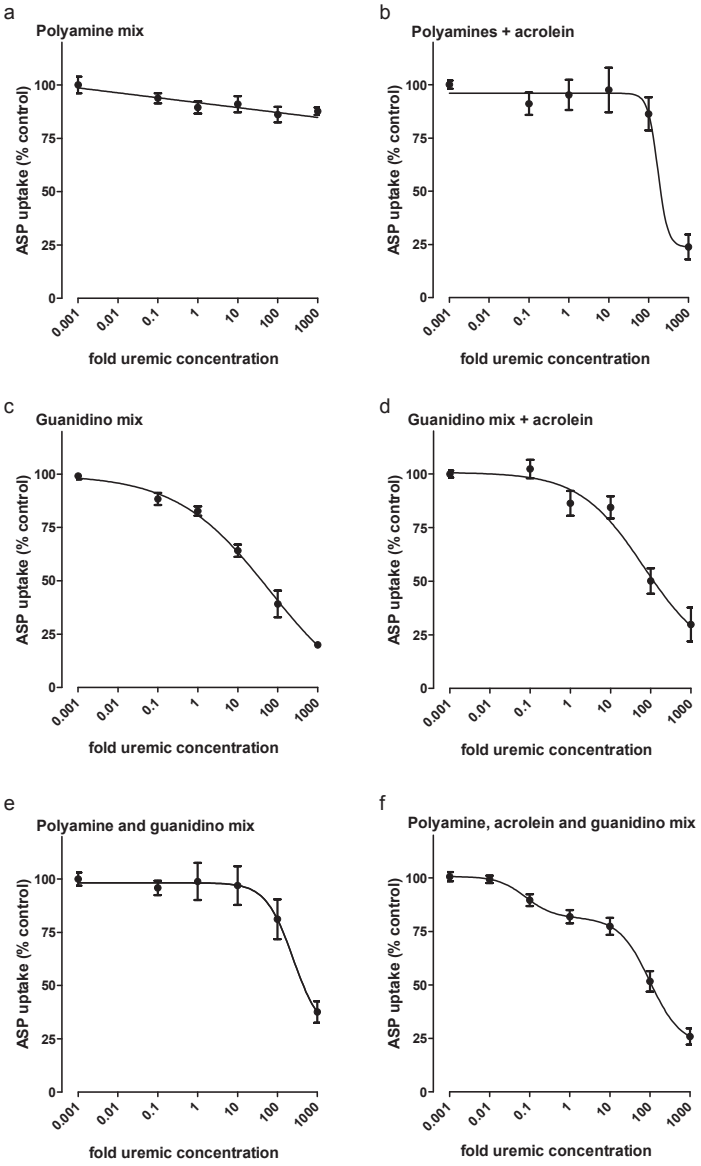


Figure 3.6 A mixture of cationic UTs inhibit ASP$^+$ uptake by ciPTEC cells at physiologically relevant concentrations. Concentration-inhibition curves of ASP$^+$ uptake by the following mixtures; polyamine mix (a), polyamines and acrolein (b), guanidino mix (c), guanidino compounds and acrolein (d), polyamines and guanidino compounds (e), polyamines, acrolein and guanidino compounds (f). Toxin concentrations were taken at 1,000, 100, 10, 1, 0.1 and 0.01 times the uremic plasma concentrations reported in literature (see Table 3.2, in µM: spermidine 0.67, spermine 0.09, cadaverine 0.21, putrescine 0.88, acrolein 1.42, guanidine 2.18, and methylguanidine 7.66). Values are given as mean ± SEM, and described as percentage of ASP$^+$ uptake in the absence of the inhibition mixture. Nonlinear regression was calculated using the GraphPad Prism sigmoidal or biphasic dose-response model. All experiments were performed at least three times in triplicate.

Table 3.2: Normal and uremic serum concentrations of the selected polyamines, acrolein and guanidino compounds reported in literature

Polyamines	C-norm (μM)	C-uremic (μM)	Ref.
Spermidine		0.67± 0.30 [b]	[29]
	0.07 ± 0.04 [b]	0.05 ± 0.02 [b]	[30]
Spermine	0.20 ± 0.05 [b]	0.09 ± 0.08 [b]	[29]
	0.031± 0.04 [b]	0.008 ± 0.007 [b]	[30]
Cadaverine	[b]	0. 21 ± 0. 11 [b]	[29]
Putrescine	0.24 ± 0.09 [b]	0.88 ± 0.31 [b]	[29]
	0.05 ± 0.03 [b]	0.09 ± 0.03 [b]	[30]
Acrolein[a]	0.53 ± 0.18[c]	1.42 ± 0.84[c]	[30]
	31 ± 9[d]	170 ± 86[d]	[30]

Guanidino compounds	C-norm (μM)	C-uremic (μM)	Ref.
Guanidine	<0.2 [b]	2.2 ± 1.2 [b]	[31]
	<0.21 [b]	2.05 ± 0.86 [b]	[32]
Methylguanidine	[b]	7.66 ± 4.38 [b]	[31]
	<0.06 [b]	2.13 ± 2.16 [b]	[32]

Values are shown as mean (C-norm); serum concentration in healthy population, and (C-uremic) highest mean uremic serum concentration in uremic patients.
a: Polyamine metabolite
b: Total serum concentration
c: Free concentration in serum
d: Protein bound concentration in serum

was found to be significant ($p<0.05$). The cytotoxic potential of these mixtures on ciPTEC was investigated further using the MTT assay (Figure 3.7). After 30 min of exposure, significant reduction in cell viability was measured only at very high concentrations corresponding to 1000 fold the reported uremic plasma concentration. No significant effect on viability was observed at lower concentrations.

DISCUSSION

In the present study, we used a unique human proximal tubule cell line to investigate the interaction between OCT-mediated transport and UTs. OCTs and, especially OCT2, is considered important in the excretion of many endogenous or xenobiotic solutes. Therefore, interference with its transport function could affect renal clearance and lead to accumulation of toxic compounds. From our study, two main novel findings could be derived. First, the

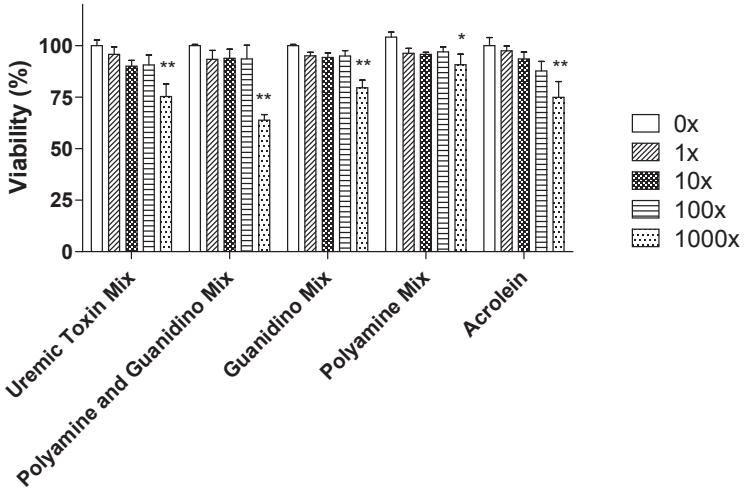


Figure 3.7 ciPTEC viability after 30 min exposure to the UT mixture.
CiPTEC were exposed to mixtures of all UTs, and mixtures of only polyamines, acrolein and the guanidino compounds. Toxin concentrations were taken at 1000, 100, 10, 1, 0.1 and 0.01 fold the uremic plasma concentrations reported in literature (see Table 3.2, in μM: spermidine 0.67, spermine 0.09, cadaverine 0.21, putrescine 0.88, acrolein 1.42, guanidine 2.18, and methylguanidine 7.66). Viability was determined by the MTT assay, performed in triplicate. Results are shown as mean values ± SEM. Significance levels * $p<0.05$, ** $p<0.01$

transport of and inhibition by known substrates and inhibitors could be confirmed in our human ciPTEC model endogenously expressing OCT2. Secondly, cationic UTs were found to inhibit cellular ASP^+ uptake. Such interaction may contribute to the progression of renal failure, as active tubular secretion is essential for the removal of cationic UTs while OCTs transport capacity is finite.

Knowledge of the clearance of cationic uremic retention solutes in humans is limited, but the human ciPTEC cell model can be a valuable tool to acquire mechanistic insights in renal cationic toxin uptake. In general, the availability of suitable human kidney cell models is scarce. A drawback of many human cell models available is their restricted endogenous transporter expression levels and functionality [33,34]. Moreover, primary renal cell cultures that are also often used in xenobiotic uptake studies quickly go into senescence [33]. With regard to cation transporters, our ciPTEC model is not hampered by these limitations. These immortalized human cells endogenously express functional OCT2 in concert with essential organic cation transporters, such as OCT1, OCT3, p-glycoprotein (*ABCB1*), breast cancer resistance protein (*ABCG2*) and multidrug and toxic compound extrusion MATE proteins (*SLC47*) (unpublished data). The co-expression of drug transporters at both the apical and basolateral site will allow for the development of transcellular transport assays. These assays

will increase our understanding of renal excretion mechanisms, superior to the study of single transporters in over expression models.

The observed increase in the V_{max} for ASP^+ uptake in matured ciPTEC corresponds to an increased mRNA and protein expression of OCT2. Therefore, we expect OCT2 to be the dominant cation uptake transporter for cationic UTs in ciPTEC. Furthermore, inhibition of ASP^+ uptake by the known substrates and inhibitors TPA, cimetidine, metformin, and quinidine, confirms functionality of OCT-mediated uptake in ciPTEC. Though some of the tested compounds can also function as substrates for OCT1 and OCT3, the apparent K_i values are in line with the values reported for other systems in which only OCT2 was over-expressed [35,36].

In the present study, the observed K_i values for the known inhibitors sometimes exceeded the measured IC_{50} values, although generally the differences are small. The variation in the numbers obtained could be influenced by, amongst others, the number of binding sites of the OCT transporters [37,38]. In the case of cimetidine, the highest concentrations of inhibitor (≥50μM) might have caused slight saturation of the system, thereby influencing the Ki values.

This investigation demonstrates that cationic UTs can interfere with ASP^+ uptake. The observed inhibitory effect of the polyamines and guanidino compounds indicate that these compounds may be able to affect uptake and hamper the clearance of xenobiotics, including exogenous toxins or drugs. The inhibition of ASP^+ transport by methylguanidine and guanidine observed here, is comparable to the findings of Kimura *et al.* who reported the interference of various guanidino-compounds with OCT2 using a human embryonic kidney cell line (HEK293) over-expressing the transporter [39]. Using the same expression system, Winter *et al.* identified putrescine as a substrate for OCT2 [40]. In agreement, our findings indicate that high concentrations of putrescine inhibit ASP^+ transport. Furthermore, Winter *et al.* observed that putrescine, spermidine, spermine and guanidine partially inhibited the accumulation of agmatine, an OCT2 and MATE substrate, in hMATE-HEK cells. Although they suggested that these compounds might influence bidirectional agmatine transport by hMATE1 [40], our findings argue that interference with OCT transporters by the polyamines might also hamper the cellular influx of these substrates and thereby limit the excretion and clearance of organic cations. Of course, since ASP^+ is a substrate for the MATE transporters as well, these efflux transporters could have influenced our measurements [41]. However, any effect of these efflux transporters on the uptake of ASP^+ would have resulted in an underestimation of maximal uptake capacities in ciPTEC. Future research should therefore be directed to trans-epithelial transport studies, in order to clarify the overall effect of the polyamines and guanidine compounds on trans-tubular organic cation transport. In Figure 3.8, the proposed mechanism of cationic toxin clearance in the proximal tubule is depicted.

Next to polyamines and guanidino compounds, we identified acrolein (a breakdown product of spermine and spermidine [42,43]) as an inhibitor of ASP^+ uptake. Out of the tested inhibitors acrolein required the lowest exposure concentration to provoke a half-maximal

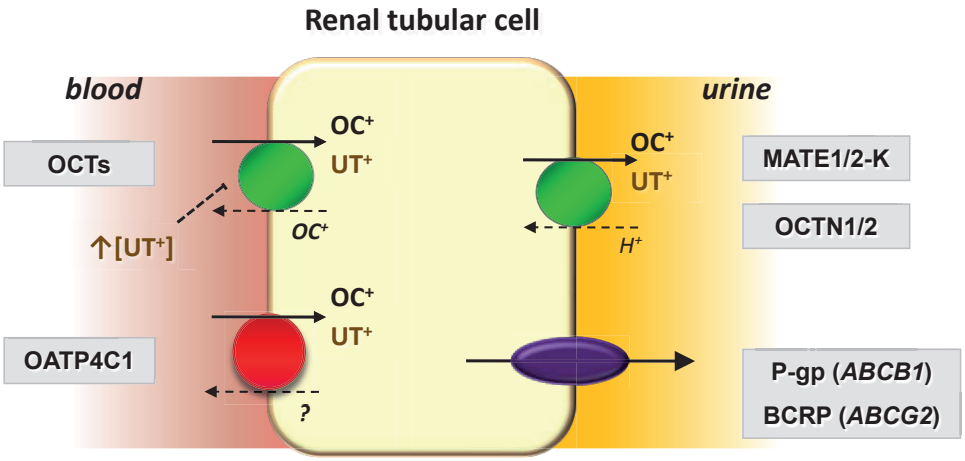


Figure 3.8 Scheme illustrating the proposed mechanisms of transport associated with organic cation (OC^+) and cationic uremic toxin (UT^+) secretion by renal proximal tubule cells.
Arrows indicate the direction of net substrate transport that occurs under physiological conditions. Solid lines depict what are believed to be the principal pathways of substrate transport; dotted lines indicate pathways that are believed to be of secondary importance. In vivo, organic cation transporters (OCTs) and the OATP4C1 transporter facilitate the uptake of cationic compounds into the cell. The MATE1/2-K and OCTN1/2 transporters facilitate the exchange of luminal H^+ for intracellular OC^+. Furthermore P-gp and BCRP mediate efflux of a wide variety of substrates. Cationic UT transport is presumed to be comparable to putative excretion of organic cations in the kidney. Furthermore, based on our findings accumulation of cationic UTs in the blood may result in inhibition of OC^+ uptake into the proximal tubule cell.

inhibition. Its K_i value lies within the range of acrolein levels that were reported in the plasma of uremic patients [42]. However, because we did not examine the effect of protein binding on ASP^+ uptake, we cannot speculate on the OCT interaction in ESRD, during which systemic protein binding of drugs and endogenous compounds can be altered [44-46].

Previously, Igarashi *et al.* showed that acrolein reduced cell growth due to cytotoxicity. Their findings suggested that accumulation of both free and protein bound acrolein in plasma of chronic kidney disease (CKD) patients could be sufficient to cause cell damage [47,48]. Indeed, we also observed decreased viability of cells exposed to acrolein, however, this was detected only at high concentrations. Therefore, we can exclude that the inhibitory effect of acrolein on ASP^+ uptake is related to its cytotoxic effect. Additionally, the Dixon plot of acrolein inhibition indicated a competitive or mixed type inhibition as the mode of interaction. Thus, it is most likely that acrolein has a direct effect on the binding site that is essential for transport, or functions as a substrate for this transporter. Furthermore, we have shown that exposure to several compounds, including acrolein, did not lead to maximal inhibition of ASP^+ uptake. The presence of OCT isoforms may explain the sub-maximal inhibition by

the UTs. To elucidate the exact mechanism, further subtype specific studies are required, for example by testing acrolein uptake in HEK cells over-expressing one specific transporter.

The systemic accumulation of UTs in CKD patients is known to negatively influence the health of the patient and contribute to the progression of renal failure. Furthermore, interactions between UTs and liver transporters, and interactions with transport proteins at the blood-brain barrier have been described [7,49]. However, to date little data are available on the exact cationic uremic toxin levels in patients. Reports on polyamine serum values in patients are not always consistent, as was recently reviewed by Duranton *et al.* [50]. For instance, whereas earlier papers reported increased spermidine levels in CKD [51], more recent publications measured decreased values in patients suffering from renal failure [30,50]. Possibly, this discrepancy is due to improvements in dialysis techniques or to methodological changes in quantification techniques.

The present findings indicate that the concentrations of individual UTs that led to inhibition of OCT transport most likely exceed the systemic levels of UTs in patients and might therefore have low clinical relevance. Nevertheless, it should be noted that in uremia, a mixture of UTs is present, and the combination of retention solutes can have cumulative, or even synergistic, effects on cation transport in the proximal tubule. Our results showed that exposure to a mixture of toxins for 30 min can cause up to 18% inhibition of ASP uptake *in vitro* when concentrations are used that reflect the situation in uremic patients. Addition of the toxins as a mixture resulted in a stronger effect as compared to the separate exposures to these toxins. Interestingly, the biphasic dose response curve was observed only when a combination of guanidine compounds, polyamines and acrolein was studied. The biphasic nature of the UTs mixture suggests different modes of action of the individual compounds or may be related to specific interaction with different subtypes of OCT transporters.

In conclusion, OCT mediated organic cation transport can be reliably studied in the ciPTEC cell model. The exposure to cationic UTs was found to negatively affect substrate uptake in proximal tubule cells. Our findings indicate that the accumulation of UTs may hamper the clearance of OCT substrates, which can also be an important factor in kidney disease progression.

ACKNOWLEDGMENTS

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4

Uremic toxins induce ET-1 release by human proximal tubule cells, which regulates organic cation uptake time-dependently

Carolien M.S. Schophuizen[1], Joost G.J. Hoenderop[2], Rosalinde Masereeuw[3,4]*, and Lambert P. van den Heuvel[1, 5]*

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Departments of [1]Pediatric Nephrology, [2]Physiology, [3]Pharmacology and Toxicology, Radboudumc, Nijmegen, The Netherlands.
[4] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, The Netherlands
[5]Department of Pediatrics, Catholic University Leuven, 3000 Leuven, Belgium

ABSTRACT

In renal failure, the systemic accumulation of uremic waste products is strongly associated with the development of a chronic inflammatory state. Here, the effect of cationic uremic toxins (UTs) on the release of inflammatory cytokines and endothelin-1 (ET-1) was investigated in conditionally immortalized proximal tubule epithelial cells (ciPTEC). Additionally, we examined the effects of ET-1 on the cellular uptake mediated by organic cation transporters (OCTs).

Exposure of ciPTEC to cationic UTs initiated production of the inflammatory cytokines IL-6 (117 ± 3%, $p<0.001$), IL-8 (122 ± 3%, $p<0.001$), and ET-1 (134 ± 5%, $p<0.001$). ET-1 exposure was accompanied by a down-regulation of OCT mediated 4-(4-(dimethylamino) styryl)-N-methylpyridinium-iodide (ASP^+) uptake in ciPTEC at 30 min (23 ± 4%, $p<0.001$), which restored within 60 min of incubation. Exposure to ET-1 for 24 h increased the ASP^+ uptake significantly (20 ± 5%, $p<0.001$). These effects could be blocked by BQ-788, indicating activation of an ET-B-receptor-mediated signaling pathway. Downstream the receptor, iNOS inhibition by (N(G)-monomethyl-l-arginine) L-NMMA acetate or aminoguanidine, as well as protein kinase C activation, ameliorated the short-term effects.

These results indicate that uremia may initiate the release of cytokines and ET-1 from human proximal tubule cells, *in vitro*. Furthermore, ET-1 exposure was found to regulate proximal tubular OCT transport activity in a differential, time-dependent, fashion.

INTRODUCTION

Renal transport processes are essential for the maintenance of body homeostasis. In the kidney, clearance of exogenous compounds and endogenous waste products from the circulation is facilitated by both glomerular filtration and active tubular secretion. The proximal tubule basolateral membrane transporters are responsible for the uptake of substrates from the circulation. While the apical transporters present in the proximal tubule facilitate their subsequent urinary release. In patients suffering from chronic kidney disease (CKD) or end-stage renal disease (ESRD), insufficient renal clearance and the subsequent accumulation of waste products lead to the development of uremia. Many solutes that accumulate in uremia have been identified [1]. However, we have only just begun to understand the effects that these compounds can exert on biological processes and their influence in the development of secondary morbidities. Previously, we demonstrated competitive inhibition of a selection of cationic uremic toxins (UTs) on organic cation uptake in a human conditionally immortalized proximal tubule epithelial cell model (ciPTEC) that endogenously expresses various renal transport proteins [2,3]. The clearance of cationic UTs (polyamines, guanidines and acrolein) largely depends on tubular secretion due to their high protein binding and/or compartmentalization [4,5]. In renal failure, the accumulation of these solutes is associated with inflammation, cardiovascular morbidity and perturbed erythropoiesis [6-8]. Moreover, conventional (hemo)dialysis methods are insufficient for their removal.

The development of a chronic inflammatory state is common in renal patients, and uremia has been identified as an important causative factor [9]. Increased levels of the vasoactive peptide endothelin-1 (ET-1), and pro-inflammatory cytokines such as IL-6, IL-8, and TNFα have been observed both before and after the start of dialysis therapy [10-14]. IL-6 promotes various inflammatory events, including the activation of lymphocytes, and is identified as a strong pro-fibrotic factor. Both IL-6 and IL-8 levels are correlated with increased mortality and poor disease outcome in renal failure [15]. TNFα is also known as an important factor in the development of renal fibrosis, and induces the production of additional inflammatory mediators such as ET-1 [16-18].

In healthy subjects, ET-1 functions as a potent peptide regulating the vascular tone, blood flow, and water and salt homeostasis. In the nephron, ET-1 mediates these processes through its tight regulatory effect on intracellular calcium, sodium and chloride channels, the production of phospho kinases and nitric oxide (NO) [19]. However, in patients suffering from renal disease, increased systemic ET-1 levels are also linked to a decline in renal function, the development of interstitial fibrosis, proteinuria, cardiomyopathy and glomerulosclerosis [20-22]. Our laboratory has previously demonstrated that exposure to nephrotoxicants can induce an ET-1-mediated signaling cascade in the proximal tubule. Upon stimulation, the proximal tubule produces ET-1 which can lead to activation of regulatory pathways including NOS and protein kinase C (PKC), ultimately resulting in a reduction of efflux transport

mediated by two apical membrane transporters P-glycoprotein (P-gp) and MRP2 [23-25]. On the other hand, long-term exposures resulted in an upregulation of the transport proteins, accompanied by nephroprotection [26,27]

In the present study, we investigated if a selection of cationic UTs, which were previously shown to interfere with tubular organic cation uptake, can act as nephrotoxicants and induce the local production of inflammatory mediators. Furthermore, we investigated if ET-1 can regulate the uptake of cationic compounds in human renal proximal tubule epithelial cells. To this end, the production of IL-6, IL-8, TNFα and ET-1 were assessed, and the short- and long-term effects of ET-1 on the regulation of organic cation influx transport in the human renal cell model were studied.

MATERIALS AND METHODS

Chemicals

Spermine, spermidine, cadaverine, putrescine dihydrochloride, acrolein, guanidine hydrochloride, methylguanidine hydrochloride, and 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) were purchased from Invitrogen (Eugene, OR, USA). Tetrapentylammoniumchloride (TPA), insulin, transferrin, selenium, tri-iodothyronine, hydrocortisone, epidermal growth factor, Endothelin-1 (ET-1), BQ788, aminoguanidine (AG), and 8-Br-cGMP were purchased from Sigma-Aldrich Co. (Zwijndrecht, the Netherlands). Sn-1,2-dioctanoyl glycerol (DOG) was obtained from Enzo Life-sciences (Raamsdonksveer, Netherlands). N(G)-monomethyl-l-arginine (L-NMMA) acetate was purchased from Tocris bioscience (Bristol, United Kingdom)

Cell culture

A previously developed and characterized immortalized human proximal tubule epithelial cell line, ciPTEC, obtained from a healthy volunteer urine samples [2,28] was cultured in Dulbecco's modified eagle medium DMEM-HAM's F12 (Lonza; Basel, Switzerland) containing 10% v/v fetal calf serum (FCS) (Greiner Bio-One; Alphen a/d Rijn, the Netherlands), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 40 pg/ml tri-iodothyronine. CiPTEC were in culture for up to 40 passages, no antibiotics or phenol red was present during this time. Since the cell model was established after immortalization using hTERT and the temperature sensitive SV40t oncogenes, the cells proliferate at 33°C and differentiate at 37°C culture conditions [2]. Regular culture was performed at 33°C 5% (v/v) CO_2, media was refreshed every 2/3 days. For experiments, cells were seeded at a density of 1:3 and left to attach for 24 h at 33° C. Subsequently, the cells were transferred to 37°C to mature for 7 days prior to the experiments.

Enzyme-Linked Immuno Sorbent Assays

To quantify the production of IL-6, IL-8, and TNFα or ET-1 by ciPTEC under various culture conditions Enzyme-Linked Immuno Sorbent Assays (ELISAs) were performed. DuoSet® ELISA Development Systems; IL-6 #DY206, IL-8 #DY208, TNFα #DY210, ET-1 #DY1160 (R&D systems, Abingdon, UK) were used to accurately measure these compounds in complete culture medium supernatant. For all assays, 500 µL of media of exposed cells was harvested after the designated incubation period. The samples were centrifuged for 5 min at 7,500xg and the supernatant stored at -20°C for a maximum of 2 months. For IL-6 and IL-8 the samples were diluted 500 times in phosphate buffered saline (PBS) or reagent diluent (PBS + 1% v/v FCS), respectively. The samples for TNFα and ET-1 were diluted 5x and 10x in PBS, respectively. The assays were subsequently performed according to the manufacturers' protocol. The optical density of each well was measured immediately using the VictorX multilable plate reader (PerkinElmer, Waltham, MA, USA) set to 460 nm. To correct for optical imperfections in the plate, the readings at 540 nm were subtracted from these measurements.

Additionally, we tested the effect of a combination of cationic UTs on cytokines and ET-1 production. To mimic uremic conditions, a mixture of toxins was used comparable to 10 or 1 times the uremic plasma concentrations reported in literature; *viz.* spermidine 0.67 µM, spermine 0.09 µM, cadaverine 0.21 µM, putrescine 0.88 µM, acrolein 1.42 µM, guanidine 2.18 µM, and methylguanidine 7.66 µM [29-32].

qPCR

Total RNA was isolated using TRIzol (Life Technologies Europe BV, Zoetermeer, the Netherlands) and chloroform extraction according to the manufacturers' protocol. 2 µg of total RNA served as a template for single-strand cDNA synthesis in a reaction using oligo (dT) and random primers in a M-MLV reverse transcriptase reaction mixture (Catalog #28025-013, Invitrogen, Bleiswijk, The Netherlands) according to the manufacturers' protocol (Doc. Rev: 100702). The mRNA expression levels were detected using gene specific primer-probe sets (Hs00174961_m1; Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems). The CFX96 Real Time PCR system (Bio-Rad Laboratories, Veenendaal, The Netherlands) was used to perform the qPCR reactions and data was analyzed using the CFX Manager™ software (Bio-Rad Laboratories). The reference gene GAPDH was used to normalize the mRNA expression levels. Data are expressed as fold increase compared to proliferating ciPTEC.

OCT mediated ASP^+ uptake

CiPTEC were cultured until confluence, in glass bottom Petri dish as described above. Cells were exposed to 100 nM ET-1 for 24 h, 30 min or taken as control. After washing the monolayer in Hepes Tris buffer (HT-Buffer: 132 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM d-Glucose, HEPES 10 mM, pH was set to 7.4 using 1.5 M Tris in MQ, 37°C)

HT-buffer containing 100 μM ASP^+ was added and the cells were incubated for 15 min at 37°C. Intracellular uptake of the fluorescent compound (dimethylamino)styryl)-N-methyl-pyridinium-iodide (ASP^+) was measured using a Zeiss Apotome Fluorescence microscope. Images were recorded over a time period of 15 min, starting 2 min after addition of 10 μM ASP^+ to enable a good focus on the cellular monolayer (Zeiss Axiovision imaging software 4.7.2). Fluorescence intensity over time was quantified for at least 6 individual cells for each condition, by plotting the Z-axis profile of the virtual stack using ImageJ software (ImageJ 1.46r, NIH, Bethesda, MA, USA).

To enable high throughput evaluation of organic cation uptake following stimulation by ET-1, in combination with pharmacological modulation of the signaling pathway, a fluorescence reader based method was used. Matured cells, cultured in 12 wells plates (seeded at approx. 150.000 cells/well) were treated with fresh medium containing the test-compounds, inhibitor or control medium for the designated incubation period. Subsequently, the cells were washed with Hepes Tris buffer (pH7.4). HT-buffer containing 10 μM ASP^+ was added and the cells were incubated for 15 min at 37°C. Next, the uptake was arrested by washing twice with ice-cold stop solution (0.5mM TPA in HT-buffer). Then the cells were lysed for 30 min lysis buffer (0.05% w/v Saponin, 0.05% v/v triton in MilliQ) and the cell homogenate transferred to a 96 wells plate (Greiner). The fluorescence measurement was performed three times at 450-642nm VictorX multilable plate reader (PerkinElmer, Waltham, MA, USA).

Data analysis

Values are given as mean ± standard error of the mean. Fluorescence levels are normalized to the unexposed control samples after subtraction of the background fluorescence at baseline (t=0). Mean values were considered to be significantly different when $p<0.05$ using a one-way ANOVA followed by Dunnett's multiple comparison test. Software used for statistical analysis was GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego CA).

RESULTS

Various cationic UTs induce IL-6, IL-8, TNFα and ET-1 production by ciPTEC

Production of the pro-inflammatory cytokines IL-6, IL-8 and TNFα by ciPTEC was measured in culture supernatant after exposure to a selection of cationic UTs (Figure 4.1A), which have previously been shown to interfere with tubular organic cation uptake. To assess the potencies of the individual UTs on cytokine production, ciPTEC were exposed to 100 μM of guanidine, cadaverine, putrescine, methylguanidine, spermine, spermidine or 10 μM of acrolein for 24 h. Acrolein was used at a lower concentration to prevent severe cytotoxicity [33]. Average baseline IL-6, IL-8 and TNF-α production by ciPTEC over 24 h were determined to be 51 ± 5.4 ng/mL, 79 ± 37 ng/mL and 227 ± 35 pg/mL respectively. Compared to controls, IL-6 production

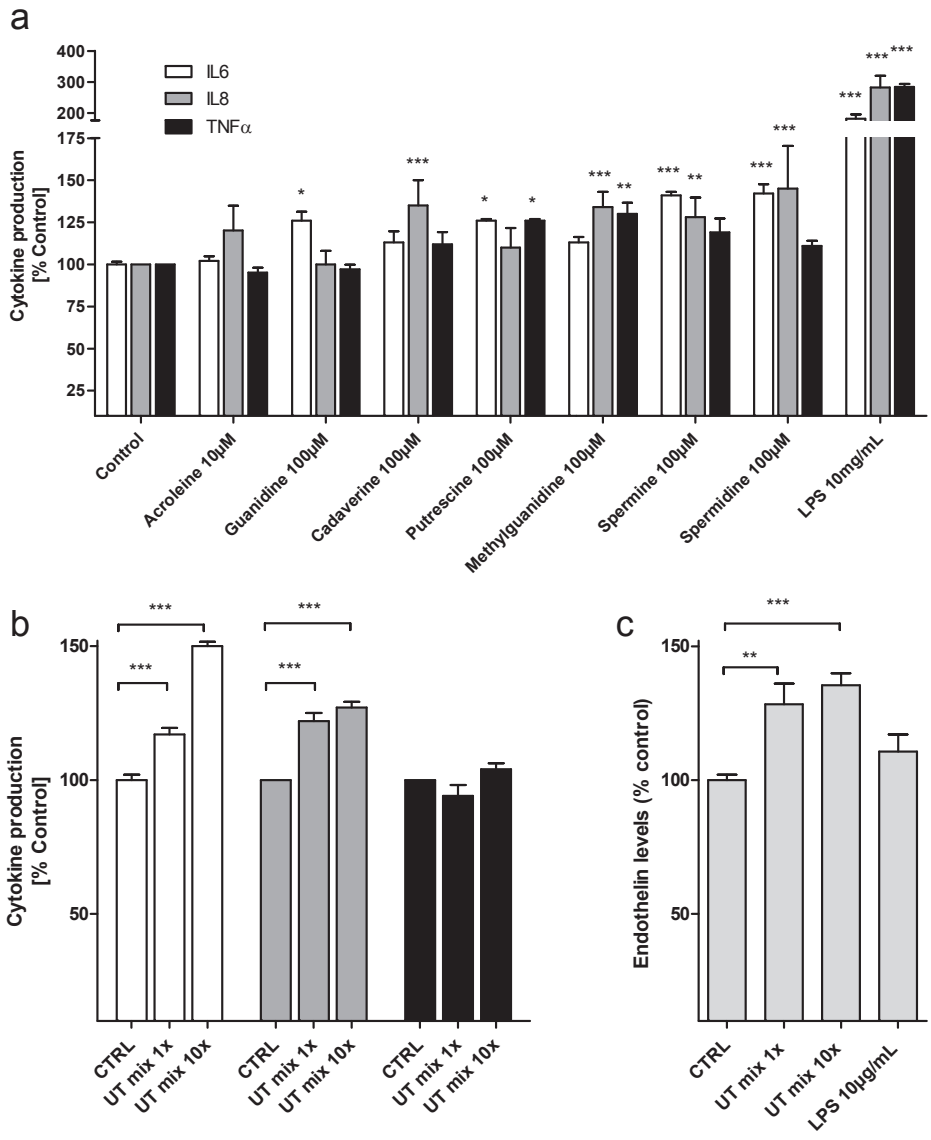

Figure 4.1: TNFα, IL-6, IL-8 and ET-1 excretions by ciPTEC after exposure to cationic UTs.
(A) TNFα, IL-6 and IL-8 excretion was measured 24 h after incubation of ciPTEC with 10 µM acrolein, 100 µM of guanidine, cadaverine, putrescine, methylguanidine, spermine, spermidine, or 10 µg/mL LPS. (B) TNFα, IL-6 and IL-8 excretion was measured 24 h after incubation of ciPTEC with a mixture of above stated cationic UTs, corresponding to 1-fold or 10-fold the levels reported in uremic patients. (C) ET-1 production by ciPTEC after 24 h of incubation with the mixture of cationic UTs or 10 µg/mL LPS. Data are expressed as the percentage of cytokines or ET-1 produced compared with untreated cells, set at 100%. Results are shown as normalized means ± SEM. For each condition at least 3 experiments were performed in triplicate (*:p< 0.05 **:p<0.01 ***: p<0.001 ANOVA).

increased significantly after stimulation by guanidine (126 ± 5%, $p<0.05$), putrescine (126 ± 1%, $p<0.05$), spermine (141 ± 2%, $p<0.001$) and spermidine (142 ± 6%, $p<0.001$). IL-8 production was stimulated by 100 μM of cadaverine (135 ± 15%, $p<0.01$), methylguanidine (134 ± 9%, $p<0.001$) spermine (128 ± 12%, $p<0.01$) and spermidine (145 ± 25%, $p<0.001$). We also detected a significant rise in TNFα production after exposure to 100 μM of putrescine (126 ± 1%, $p<0.05$) and methylguanidine (130 ± 7%, $p<0.05$; Figure 4.4.1A). Lipopolysaccharide (LPS, 10 μg/mL) was used as a positive control, which caused a significant increase in IL-6 (181 ± 14%, $p<0.001$), IL-8 (282 ± 23%, $p<0.001$) and TNFα levels (284 ± 9%, $p<0.001$).

When ciPTEC were exposed to a mixture of the selected cationic UTs for 24 h (i.e. toxin concentrations corresponding to those found in uremic patients, see methods section for actual concentrations used; a significant increase in IL-6 (117 ± 3%, $p<0.001$) and IL-8 (122 ± 3%, $p<0.001$) cytokine levels could be detected (Figure 4.1B). When increasing the UT mixture to 10-fold the concentrations reported in patients, IL-6 and IL-8 levels increased even further, until 150 ± 2% and 127 ± 2% ($p<0.001$) when compared to control cells, respectively. TNFα levels did not rise upon exposure to these uremic mixtures.

Production of the vasoactive peptide and inflammatory mediator ET-1 by ciPTEC was measured in culture supernatant after exposure to the mixture of the selected cationic UTs for 24 h. Figure 4.1C depicts the ET-1 release by ciPTEC. Average baseline ET-1 production by ciPTEC over 24 h was determined to be 14.6 ± 4 pg/mL (11 ± 4 pg/mg protein). Exposure to the 1-fold and 10-fold cationic UT mixture for 24 h increased ET-1 production by ciPTEC, up to 128 ± 7% ($p<0.01$) and 134 ± 5% ($p<0.001$), respectively. Exposure of ciPTEC to the individual cationic uremic compounds for 24 h did not lead to significantly affected ET-1 release (data not shown). Remarkably, LPS did not induce ET-1 production by ciPTEC.

Differential effect of ET-1 exposure on organic cation transport.

To investigate if ET-1 could affect the functionality of the organic cation transporters (OCT) present in ciPTEC, we measured the intracellular uptake of the fluorescent compound ASP^+ after exposure to ET-1. A dose-response curve showed a significant increase in ASP^+ uptake at concentrations of 100 nM and 1 μM ET-1 (Supplemental figure S1). As a control, TPA was used which inhibited ASP uptake by approx. 70%. In Figure 4.2, representative fluorescence images (A) and their quantified fluorescent signals (B) are shown for the ASP^+ uptake by ciPTEC in the presence or absence of 100 nM ET-1 exposure. A significant increase in maximal uptake was observed after 24 h of incubation, while 30 min of exposure diminished the uptake compared to unexposed cells. By using a fluorescent reader based method, more conditions were tested in a high throughput fashion (Figure 4.3A). Again, after 30 min of pre-incubation with 100 nM ET-1, a 23 ± 4% ($p<0.001$) decrease in ASP^+ uptake was measured. This effect was not observed after increasing the pre-incubation time to 3 or 6 h. However, after 24 h of pre-incubation, a 20 ± 5% ($p<0.001$) increase in ASP^+ uptake was observed. Addition of the ET-1 receptor inhibitor BQ-788 during the exposures normalized the ASP^+ uptake levels for all time points (Figure 4.3B).

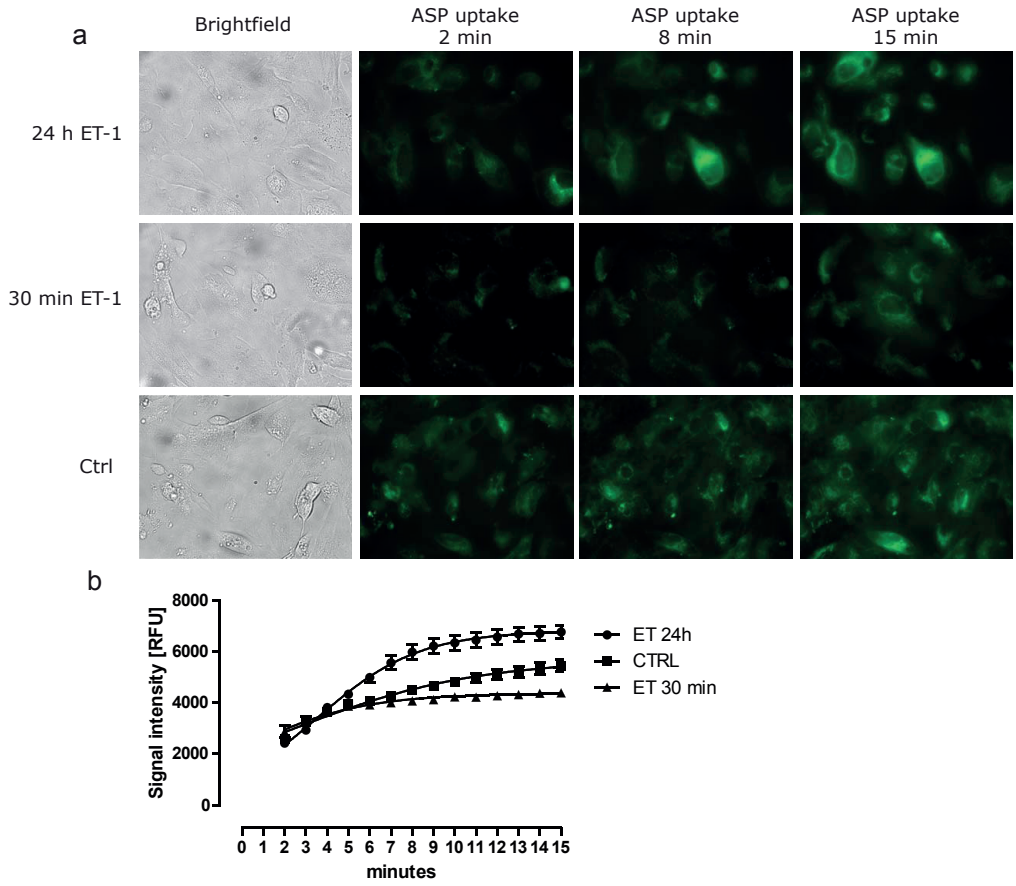

Figure 4.2: Changes in ASP$^+$ uptake by ciPTEC in response to exposure to ET-1.
CiPTEC were incubated for 30 min or 24 h with 100 nM ET-1 before measuring the intracellular uptake of the fluorescent cationic compound ASP$^+$ (10 μM). (A) Representative confocal images of 24 h ET-1 exposed (top row), 30 min ET-1 exposed (middle row) or control cells (bottom row) taken at 2, 8 or 15 min after addition of ASP$^+$. (B) ASP$^+$ accumulation in cells was determined over the 15 min time period for 24 h ET-1 exposed (●), 30 min ET-1 exposed (▲) or control cells (■). Fluorescence intensity over time was quantified for at least 6 individual cells for each condition. Images were captured by real time imaging. Prior to addition of the fluorescent compound, brightfield images (panel A) were taken. The addition of the fluorescent substrate resulted in minor cell movement, thereby affecting cell positioning to some extent.

In humans, OCT2 (SLC22A2) is considered one of the most important renal OCT [34], we examined its mRNA expression levels after exposure to 100nM ET-1 for 30 min or 24 h and compared these results to the control situation. Additionally, the expression levels of OCT1 and OCT3 were examined as these are expressed in ciPTEC as well [3,35]. Exposure to

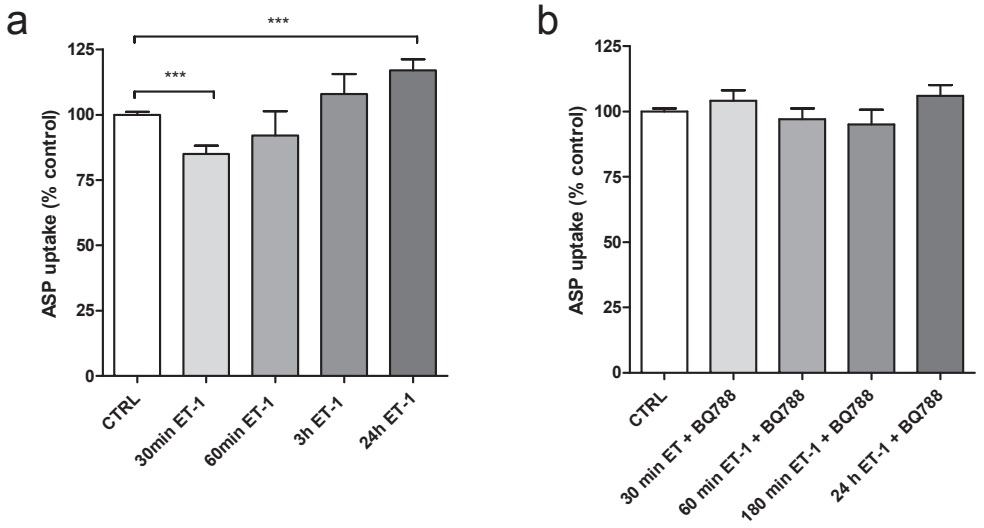

Figure 4.3: Regulatory effects on OCT mediated ASP$^+$ uptake through ET-1 Receptor.
Pre-incubation of ciPTEC with 100 µM ET-1 for 30 min up to 24 h inhibits the cellular uptake of 10 µM of the fluorescent substrate ASP$^+$. (A) The effects of 100 µM ET-1 on steady state (15 min) ASP$^+$ accumulation. (B) ASP$^+$ accumulation of ciPTEC after pre-incubation with a combination of 100 nM ET-1 with the ET-B receptor inhibitor BQ788 (1µM), which blocked the effect of ET-1 at all time points. Values are depicted as means ± SEM for at least 3 experiments performed in triplicate (*:p< 0.05 **:p<0.01 ***: p<0.001).

ET-1 did not significantly alter OCT1, 2 or 3 expression levels on short or long term (Supplemental figure S2).

iNOS inhibition attenuates the inhibitory effects of ET-1 exposure on organic cation uptake

Previous studies demonstrated that ET-B receptor activation can initiate an NO mediated signaling pathway that regulates drug transport in the proximal tubule [24]. Therefore, we investigated if the differential regulation of intracellular organic cation uptake induced by ET-1, resulted from nitric oxide synthase (NOS) activation. ciPTEC were pre-incubated with 100 nM ET-1 in the absence or presence of the nonselective NOS-inhibitor l-arginine analogue N(G)-monomethyl-l-arginine (L-NMMA). Figure 4.4A shows that L-NMMA alone did not influence ASP$^+$ uptake by ciPTEC, but attenuated the ET-1 mediated down-regulation in ASP$^+$ uptake at 30 min. This effect was not observed after the 24 h pre-incubation period. Pre-incubation with the selective inducible NOS inhibitor AG in the presence or absence of ET-1 produced similar results, attenuating only the short-term ET-1 mediated effect (Figure 4.4B).

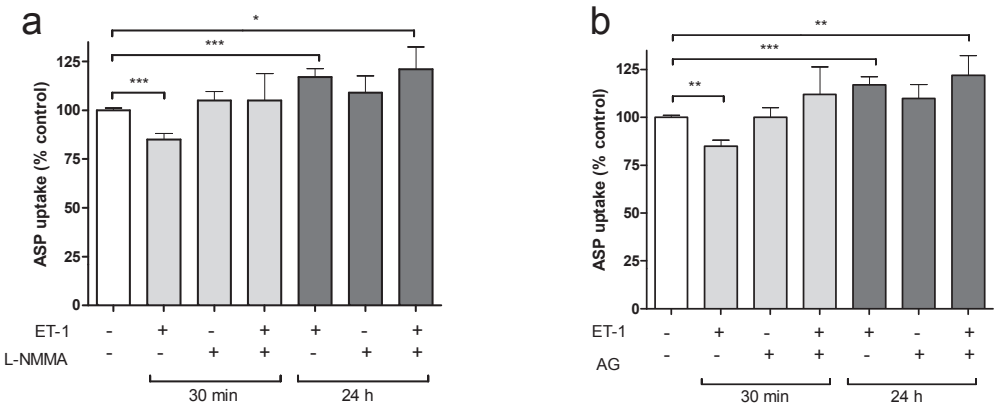

Figure 4.4: NOS-inhibition reverses the short-term action of ET-1 on ASP$^+$ uptake.
CiPTEC monolayers were incubated for 15 min in medium containing 10 µM ASP$^+$ without or with pre-incubation with (A) 100 nM ET-1, 100 µM L-NMMA or ET-1 plus L-NMMA, (B) 100 nM ET-1, 100 µM aminoguanidine (AG) or ET-1 plus 100 µM AG. Subsequently, the reaction was stopped, the cells were lysed and the fluorescent signal was measured as described in the methods section. Values are presented as means ± SEM for at least 3 experiments performed in triplicate. (*:p< 0.05 **:p<0.01 ***: p<0.001).

PKC activation restores short-term organic cation uptake in ciPTEC after ET-1 exposure

Cyclic guanosine monophosphate (cGMP) activation was simulated by the addition of 1 µM of 8-br-cGMP, a cell permeable analog of cGMP, which was previously identified as an important signaling molecule in ET-1 regulated MRP2 transport in the proximal tubule [36]. After pre-incubation of ciPTEC with 1 µM 8-Br-cGMP alone, the intracellular ASP$^+$ levels did not differ from the control conditions (Figure 4.5A). Also, combining the pre-incubation to ET-1 exposure with 8-Br-cGMP could not prevent either the inhibitory effect on ASP$^+$ uptake at 30 min, or the stimulation observed after 24 h. These results point towards an absence of protein kinase G and cGMP as signaling molecule in this ET-1-mediated pathway. Protein kinase C was stimulated by pre-incubation with the PKC activator sn-1,2-dioctanoyl glycerol (DOG), which is a cell permeable analog of the PKC-activating second messenger diacylglycerol (DAG). Pre-incubation of ciPTEC with 1 µM DOG alone did not alter ASP$^+$ uptake compared to the non-exposed control situation (Figure 4.5B). Combining ET-1 and DOG during 30 min of pre-incubation restored the ASP$^+$ uptake by ciPTEC to the control situation. This effect of DOG was not observed after 24 h of ET-1 incubation, suggesting that two separate pathways regulate organic cation transport.

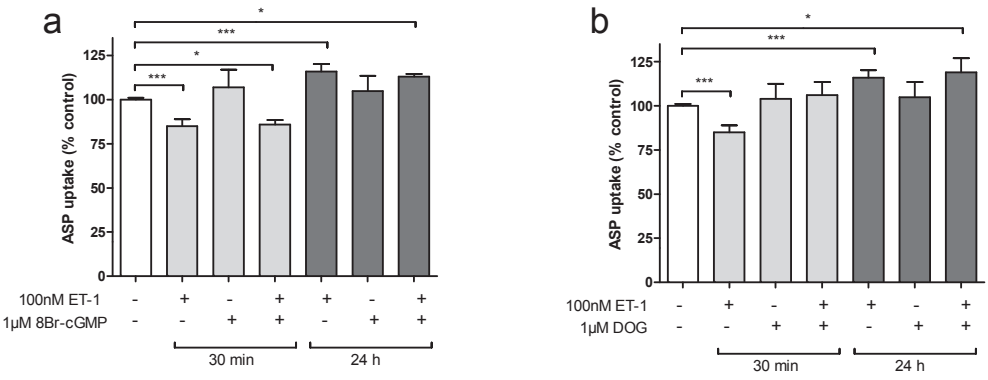

Figure 4.5: Kinase pathways on ciPTEC ASP$^+$ uptake levels after exposure to ET-1.
ciPTEC monolayers were incubated for 15 min in medium containing 10 μM ASP$^+$ without or with pre-incubation with (A) 100 nM ET-1, 1 μM DOG or ET-1 plus DOG, (B) 100 nM ET-1, 1 μM 8-br-cGMP or ET-1 plus 100 μM 8-br-cGMP. Subsequently, the reaction was stopped, the cells lysed and the fluorescent signal was measured as described in the methods section. Values are expressed as means ± SEM for at least 3 experiments performed in triplicate. (*:p< 0.05 **:p<0.01 ***: p<0.001).

DISCUSSION

The results of the present study indicate that exposure of human proximal tubule epithelial cells to cationic UTs leads to cytokine and ET-1 production by ciPTEC. We demonstrated that the production of IL-6, IL-8, and TNFα was stimulated by the exposure to high levels of individual cationic toxins, supporting the inflammatory response often observed in uremia. At clinically relevant concentrations, a mixture of these selected toxins induced the endogenous production of IL-6, IL-8 and ET-1. Additionally, ET-1 was identified as time-dependent regulator of organic cation uptake through interaction with the ET-B receptor.

In patients suffering from renal failure, the accumulation of UTs was reported to be associated with the development of a chronic inflammatory state [9]. Various clinical studies have demonstrated a correlation between increasing IL-6, IL-8 and TNFα cytokine levels and reduced glomerular filtration rate [11,12]. Inefficient clearance of the inflammatory mediators in combination with increased cytokine production could cause these effects. The results of the present study confirm that exposure to a cationic uremic mixture of acrolein, spermine, spermidine, cadaverine, putrescine, guanidine and methylguanidine induced the production of IL-6 and IL-8 by using the human renal cell line, ciPTEC.

The observed increase in IL-6 and IL-8 production ranged from 26-45%. When compared to the 81-182% increase in IL-6 and IL-8 release after LPS stimulation this level could be considered modest. However, LPS is one of the most potent inducers of cytokine production. Patients suffering from ESRD were reported to show a 35% rise in IL-6 levels [11]. The local

production of these cytokines in response to a uremic milieu can promote local profibrotic or inflammatory processes *in-vivo*, since IL-6 production is associated with the induction of fibrotic gene expression, and IL-8 is a potent neutrophil chemoattractant [37,38]. We could therefore consider the 26-45% increase in production of IL-6 and IL-8 by proximal tubule epithelial cells clinically relevant. It is, however, important to take into account that our in vitro settings cannot be translated directly to the clinical situation, especially with regard to protein binding and intracellular solute concentrations. Not only are organic solutes often able to bind to human serum albumin, they can also interact with other proteins such as α1-acid glycoprotein and lipoproteins, and even red blood cells and platelets [39]. On the other hand, it is known that ESRD can affect systemic protein binding of drugs and endogenous compounds due to displacement [40-42]. Protein binding could, therefore, affect the free fraction of uremic solutes available to cells. Nevertheless, intracellular levels often easily exceed the free serum concentrations due to active uptake processes[43,44].

The absence of TNFα production by ciPTEC after exposure to the UT mixture contrasts to the effects of the individual compounds putrescine and methylguanidine. In the mixture, the concentrations of these compounds were much lower as compared to the experiments performed with the individual compounds, which might explain this effect. Additionally, TNFα is known to possess a very short half-life in whole blood [45], and low concentrations are reported to denature rapidly in culture medium at 37°C [46,47]. Since ciPTEC were exposed for 24 h, the initial TNFα peak could have subsided before the measurement was performed.

Many cytokines, including IL-6, are known to stimulate ET-1 production. Furthermore, the proximal tubule can endogenously produce ET-1 upon stimulation with various nephrotoxicants, as was previously demonstrated in non-human species [24,48,49]. Here we show that, next to the production of IL-6 and IL-8, the mixture of cationic UTs also promoted a modest ET-1 release by ciPTEC. The local production of ET-1 in the renal tubule has been recognized as an early response to tubular injury, and was identified as a key regulator of efflux transporters, Mrp2 and P-gp [23,26,36,48-50]. In these studies the threshold for ET-1 action on transporters was determined between 0.5-10 nM (1.25-24.9 ng/mL). In our study, the ET-1 release by ciPTEC was well below this threshold (Figure 4.1). The quantity of hormone released by ciPTEC is small, and much diluted by the cell culture medium. However, since the cells produce this compound endogenously, the threshold level could be reached in the direct vicinity of the cells.

The results of the present study also demonstrate that next to the regulation of MRP2 and P-gp, ET-1 can differentially regulate cation uptake in ciPTEC. Because ET-1 is a hydrophilic compound, surface receptors are necessary to regulate intracellular responses to the peptide. The regulatory effects of ET-1 on the OCT-mediated ASP^+ uptake were clearly mediated through the action of the ET-B-receptor, since the addition of the ET-B-receptor blocker BQ-788 ameliorated both effects observed after 30 min and 24 h.

The inhibition of OCT-mediated ASP^+ transport by ET-1 after 30 min is in line with earlier reports. Terlouw *et al.* previously hypothesized that the reduction in efflux activity by transporters could protect the cell form injury after exposure to a nephrotoxic substance by saving ATP for more immediate processes that are necessary for cell survival [24,49]. Although the reduction in ASP^+ uptake by ciPTEC after 30 min suggests a similar protective mechanism for organic cations, the reduced influx is unlikely to serve as an ATP saving mechanism. Organic cation uptake processes are mostly mediated by polyspecific solute carriers like OCT2 (*SLC-22* family), which are membrane potential and substrate concentration gradient dependent, and therefore functions as facilitated diffusion carriers for organic cations, but are independent of proton gradients. In addition, two other types of apically expressed transporters might be involved, viz. the Carnitine/Organic Cation Transporters OCTN1 and OCTN2 (SLC22A4 and SLC22A5 [51,52]) and the proton antiporters, MATE1 and MATE2k (multi-antimicrobial extrusion proteins). Schmidt-Lauber et al. reported active uptake of ASP^+ through OCTN1 and MATE1 in transporter-transfected HEK293 cells and human synovial fibroblasts (hRASF) [53]. In ciPTEC, the exact role of these transport proteins in the uptake of cationic organic substrates, such as ASP^+ or uremic retention solutes, is yet unclear. Future studies should be directed to further elucidate their role.

Solute carriers do not rely directly on ATP binding or hydrolysis [54,55]. Based on our results, we suggest that the short-term reduction in organic cation influx by ET-1 signaling provides protection by minimizing intracellular accumulation of potentially toxic compounds. In acute situations, this would reduce intracellular damage until other clearance processes are activated to handle the toxic threat. On the other hand, the increased influx transport observed after 24 h was unexpected. This observation could not be linked to increased mRNA levels of OCT2 neither OCT1 or OCT3, of which detection on mRNA level in ciPTEC was remarkable, since they are generally considered of less importance in human kidney [34,35] (supplemental figure S2).We cannot exclude the involvement of another important mechanism in the short-term regulation of transporter activity known as endocytic membrane retrieval, or insertion as suggested previously [56,57]. This mechanism has also been described in the short-term regulation of the proximal tubular type IIa Na-P_i cotransporter, and the pH-regulated insertion of H^+-ATPases in the proximal tubule [58-61]. Furthermore, rapid insertion of MRP2 in response to tubulotoxic insults has been described [26,62]. If OCT functionality is regulated by such rapid dynamic endocytic retrieval or insertion processes, the regulation depends on an intracellular vesicular pool of transporters and its regulation will therefore not directly affect mRNA expression levels. The development of specific antibodies directed at the human organic cation transporters, together with immunocytochemisty or protein expression studies, could in the future provide more insight in the involvement of the regulatory mechanisms of organic cation uptake in ciPTEC. Furthermore, post-translational modifications, such as phosphorylation could affect transporter activity. In the intracellular loops of the (human) *SLC-22* family, several potential phosphorylation sites have been identi-

fied [63]. For rOCT1, phosphorylation events are known to stimulate conformational changes at the substrate binding site, thereby increasing the affinity for its substrates [64]. In various studies focusing on ASP^+ uptake in rabbit or human models, PKC stimulation was found to either induce or inhibit substrate uptake, depending on the species [65].

In ciPTEC, PKC stimulation by DOG in combination with ET-1 restored organic cation uptake after 30 min of pre-incubation. These observations suggest that PKC activation in ciPTEC stimulates organic cation uptake, however, this is in contrast to previous studies with hOCT2-HEK293 cells and isolated human proximal tubules [57,65]. The reason for this discrepancy is unclear, but considering the many intracellular processes affected by PKC this may suggest that multiple regulatory pathways are involved. Still, the findings of the present study are in agreement with the known interaction between ET-1 and the protein kinase pathway in the regulation of other renal transporters (viz. Mrp2 and P-gp) [23,66,67].In killifish renal proximal tubules, dogfish shark salt glands and rat brain capillaries, ET-1 also reduced Mrp2 or P-gp-mediated transport by stimulating the PKC pathway . It would, therefore, be interesting to evaluate in future studies how ET-1 production by ciPTEC would affect the transport by Mrp2 or P-gp in this cell model.

Next to the PKC pathway, our results demonstrated that inhibition of iNOS by AG or L-NMMA restored ASP^+ uptake at 30 min after ET-1 exposure. In the human proximal tubule, iNOS is constitutively expressed [68,69]. It mediates the regulation of local inflammatory responses, following cytokine production during endotoxemia, or after exposure to nephrotoxicants [48,49]. Similar to the iNOS induced effects observed in the present study, Heemskerk *et al.* demonstrated a reduction in OCT1 and OCT2 uptake transport during acute endotoxemia, while the functional expression of the efflux transporters MRP2 and P-gp, increased [25,70]. Therefore, iNOS is considered an important player in the regulation of the short-term tubular response to external assaults. These short-term effects of the inflammatory mediator ET-1 on the regulation proximal tubular cation uptake could provide clues on possible pathways involved in acute kidney injury.

Though numerous studies have investigated the mechanisms involved in renal proximal tubular transport, the mechanisms behind these regulatory pathways is often analyzed within relatively short time frames. We observed a time-dependent ET-B receptor mediated effect of ciPTEC exposure to ET-1, leading to an increased cellular uptake of cationic substances. The mechanisms behind this long-term (24 h) regulation could not be explained by the known NO, PKC or cGMP mediated pathways. These observations are reminiscent of both the biphasic and NO-independent pathways that were described for the efflux transporter P-gp [71,72], for which a second pathway, next to NO mediated regulation, was identified involving activation of Toll Like Receptor 4 and translocation of NF-κB. Further research would be warranted to investigate this pathway in the regulation of proximal tubular organic cation uptake. Investigating the increase in substrate uptake following long term ET-1 exposure

might provide clues about possible treatment strategies to promote renal secretory clearance in uremia, or shed further light on protective mechanisms of the renal proximal tubule.

In conclusion, our findings implicate that cationic UTs can directly induce the local production of inflammatory cytokines and ET-1. Furthermore, ET-1 exposure was found to enable regulation of organic cation uptake by proximal tubule cells. These findings might suggest that systemic accumulation of UTs in patients suffering from CKD could influence renal tubular clearance processes through local production of inflammatory or vasoactive mediators. However, further studies on OCT regulation and functionality in response to UT exposure are required to elucidate the exact processes that connect these events.

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SUPPLEMENTARY DATA

Supplementary data is available at http://www.mdpi.com/2073-4409/4/3/234

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Development of a living membrane comprising of a functional human renal proximal tubule cell monolayer on polyethersulfone polymeric membrane

Schophuizen CMS[1,2,3]*, De Napoli IE[4]*, Jansen J[1,2,3], Teixeira S[4], Wilmer MJ[3], Hoenderop JGJ[2], van den Heuvel LP[15], Masereeuw R[3], Stamatialis D[4]

* Authors contributed equally

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Departments of [1]Pediatric Nephrology, [2]Physiology, and [3] Pharmacology and Toxicology, Radboudumc, Nijmegen, The Netherlands.
Department of [4]Biomaterials Science and Technology, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, The Netherlands
Department of Pediatrics[5], Catholic University Leuven, Leuven, Belgium.

ABSTRACT

The need for improved renal replacement therapies is a stimulance for innovative research for the development of a cell based bioartificial kidney, or so called Renal Assist Device (RAD). A key requirement for such device is the formation of a "living membrane" consisting of a tight kidney cell monolayer with preserved functional organic ion transporters, on suitable artificial membrane surfaces. In this work, we applied a unique conditionally immortalized proximal tubule epithelial cell (ciPTEC) line with an optimized coating strategy on polyethersulfone (PES) membranes to develop a "living membrane" with a functional proximal tubule epithelial cell layer. PES membranes were coated with combinations of 3,4-dihydroxy-L-phenylalanine and human Collagen IV (Coll IV). The optimal coating time and concentrations were determined to achieve retention of vital blood components while preserving high water transport and optimal ciPTEC adhesion. The ciPTEC monolayers obtained were examined through immunocytochemistry to detect zona occludens-1 (ZO-1) tight junction proteins. Reproducible monolayers were formed when using a combination of 2 mg/ml L-DOPA (4 min coating, 1 h dissolution) and 25 μg/ml Coll IV (4 min coating). The successful transport of ^{14}C-creatinine through the developed "living membrane" system was used as an indication for organic cation transporter functionality. Addition of metformin or cimetidine significantly reduced the creatinine transepithelial flux, indicating active creatinine uptake in ciPTEC, most likely mediated by the organic cation transporter, OCT2 (*SLC22A2*).

In conclusion, this study shows the successful development of a "living membrane" consisting of a reproducible ciPTEC monolayer on PES membranes, an important step towards the development of a bioartificial kidney.

INTRODUCTION

The number of patients with end-stage renal disease (ESRD) is progressively increasing and the need for renal replacements therapies is rising [1]. Worldwide, over 2 million patients suffer from ESRD and each year that number grows by 5%. Since transplant options are limited [2], approximately 70% of patients receive (hemodialysis or peritoneal) dialysis treatment. While dialysis therapy has already prolonged many ESRD patients' lives, the treatment cannot completely replace renal function. Mortality (15-20% per year) and morbidity of these patients remain high, whereas their quality of life is generally low. Hemodialysis (HD) therapy removes mainly small, unbound substances from the circulation, while leaving large, compartmentalized and protein-bound uremic retention solutes untouched [3]. Although the introduction of high-flux (HF) or hemodiafiltration (HDF) therapy promotes enhanced removal of middle molecular weight solutes, the removal of protein-bound retention solutes remains limited [4,5].

Since renal secretion of such protein-bound compounds is predominantly mediated by the proximal tubule epithelial cells (PTEC) [6,7], the need for improved renal replacement therapy has stimulated innovative research into the development of a bioartificial kidney, or so called Renal Assist Devices (RAD) [8]. These innovative devices aim to complement the hemodialysis treatment, by coupling artificial membranes with functional kidney cells. To achieve efficient clearance of uremic retention solutes, proximal tubular epithelial cells need to be seeded on the inner surface of hollow fiber membranes. This situation is comparable to the native tubule, where the basement membrane separates proximal tubule cells from the circulation. While the patients' blood flows over the outer surface, the uremic retention solutes can bind to the transport proteins present on the basolateral surface of human PTEC, facilitating their subsequent secretion into the hemodialysis filtrate. This approach could provide a means to further diminish the systemic accumulation of uremic retention solutes, reduce the incidence of secondary morbidities in patients, and shorten dialysis duration or frequency, thereby improving the quality of life in ESRD.

The development of a RAD brings along very specific biotechnological challenges, as was recently reviewed by Jansen et al. 2014 [9]. First of all, an important challenge in the development of a RAD is the limited availability of reliable and well characterized human proximal tubule cells capable of transepithelial excretion of uremic retention solutes. This "living membrane" should consist of tight cellular monolayers maintaining their typical polarity and functionality. Second, one side of the membrane to be used in a RAD needs to be suitable for blood contact (low cell adhesion), whilst the other side should facilitate cellular adhesion. Surface topography was identified as an important feature affecting cell adhesion and membrane hemocompatibility, the two properties being favored by higher surface roughness and smoothness, respectively [10]. Several studies, which attempted to create "living membranes" with renal epithelial cells, have used existing membranes (mostly hollow

fibers) used in dialysis and / or blood purification. Polyethersulfone (PES), polysulfone (PSF), polyacrylonitrile (PAN) and cellulose acetate membranes, were evaluated for their ability to support monolayer growth of renal epithelial cells [11-13]. Since these membranes are designed for low cell adhesion, and maximum hemocompatibility during blood filtration, it is necessary to apply a coating consisting of extracellular matrix components. This actually describes the third challenge in RAD development; the optimization of coating conditions. In some cases, coatings have been reported to improve the attachment of renal tubule epithelial cells, but often coatings failed to maintain trans-monolayer transport activity and monolayer integrity [14-18]. Human collagen type IV (Coll IV) based coatings are promising for attachment and functional culture of proximal tubule cells [15], since Coll IV is endogenously present in the basal lamina and promotes mesenchymal cell differentiation towards the epithelial lineage [19]. However, it is necessary to improve its adhesion to the artificial membrane. The treatment of polymeric surfaces with biomolecules via covalent reactions is generally inconvenient given the susceptibility of different activated groups to hydrolysis, which can lead to low efficiency of surface biofunctionalization [20]. An easy two-step water based biofunctionalization method has been proposed that promotes the formation of a thin adherent polydopamine (PDA) film [10,17,21] using self-polymerizing 3,4-dihydroxy-L-phenylalanine (L-DOPA); and exploits the reactivity of the PDA film to covalently bind the biomolecule Coll IV on the membrane surface [10,20]. However, in these earlier studies, the application of the coatings was not optimized with regard to membrane transport characteristics (high water permeance and retention of vital proteins such as BSA and IgG).

The selection of renal cell lines suitable for RAD application poses another challenge. The use of animal cell models, such as porcine (primary or LLC-PK1), monkey (JTC-12) and canine (MDCK) renal epithelial cells, provides insight in the functionality of a RAD, both *in-vitro* as well as *in-vivo* [13,16,22-25]. However, their clinical application is highly restricted. Moreover, species differences in renal proximal tubule physiology are in favor of a renal cell line of human origin [26,27]. Studies using freshly isolated primary human proximal tubule cells in a RAD have shown promising results [10,28,29]. However, their limited availability, low proliferative capacity, and donor-to-donor variation, severely restrict the use of these cells. The utilization of a functional human proximal tubule epithelial cell line overcomes these drawbacks. Previously, some of the co-authors of this study have developed a human conditionally immortalized proximal tubule epithelial cell (ciPTEC) line, with intact proximal tubular characteristics and endogenous expression of various functional transport proteins [30,31]. Among the advantages of this cell line is the switch between a proliferative state at 33 °C to a differentiation state at 37 °C, permitted by transduction with the temperature sensitive Simian virus 40 (SV40) large T antigen. The genetic switch allows for cell proliferation arrest when the cells are confluent and fully differentiated, promoting the formation of tight and functional monolayers [30].

In this work, we show for the first time the development of a stable, functional "living membrane" by culturing ciPTEC on PES based membranes with a molecular weight cut off 50 kDa. This cut off prevents albumin leakage, blocks possible immunoglobulin transfer, and avoids the loss of vital blood component in case of cell monolayer integrity failure. The membranes were coated with a double coating consisting of L-DOPA and Coll IV on the more porous rough membrane side to maximize the beneficial effect of both membrane topography and coating on cell adhesion. This coating was carefully optimized to preserve high membrane permeability and create a tight cell monolayer. The selective active transport of creatinine through the "living membranes" was investigated in the presence and absence of specific inhibitors, and compared to a commercially available polyester Transwell® system.

MATERIALS AND METHODS

Membrane coatings

Flat polyethersulfone membranes (Sartorius, Germany), which showed good hemocompatibility properties [32], with a molecular weight cut off of 50 kDa (indicated, as PES - 50) were coated with a double layer of 3,4-dihydroxyl-L-phenylalanine (L-DOPA) (D9628, Sigma Aldrich, Zwijndrecht, The Netherlands) and human collagen IV (Coll IV, C6745, Sigma Aldrich, Zwijndrecht, The Netherlands) L-DOPA solutions with concentrations ranging from 1 to 2 mg/ml in Tris buffer 10 mM (pH 8.5) were prepared and dissolved at 37°C. Complete dissolution was obtained after 1 h, followed by filter sterilization. Subsequently, the L-DOPA solution was used to either coat the membranes immediately after powder dissolution, with minimum polymerization degree (referred to as L-DOPA (1 h)), or left to polymerize for 2 more hours (referred to as L-DOPA (3 h)) before coating the membranes. For the sake of simplicity, we defined the dissolution time as the time that the L-DOPA is kept in solution before coating the membrane. The membranes were first pre-incubated in Hanks' balanced salt solution (HBSS; Life Technologies, Bleiswijk, the Netherlands) for 10 min. Next, the L-DOPA solutions were applied to the membrane surface. The solution was aspirated, and the membranes were left to dry for 5 min. Then, a Coll IV solution (25 μg/ml or 50 μg/ml) in HBSS was used for the second step of the coating. After fluid aspiration and drying, the membranes were washed three times in HBSS to remove any remaining solvent or unbound compound. Diverse L-DOPA dissolution times (1 and 3h) and coating times of membranes (1 and 4 min) were tested and their effects on membranes properties were evaluated in terms of membrane morphology and pure water permeance. An overview of the coating conditions tested is given in Figure 5.1. The PES-50 membrane has an asymmetric structure: one membrane side is denser (and therefore determines the transport properties) and smooth, while the other membrane side is more porous and rough. The double coating was performed on the more porous membrane side, under static conditions, by contacting the coating solution

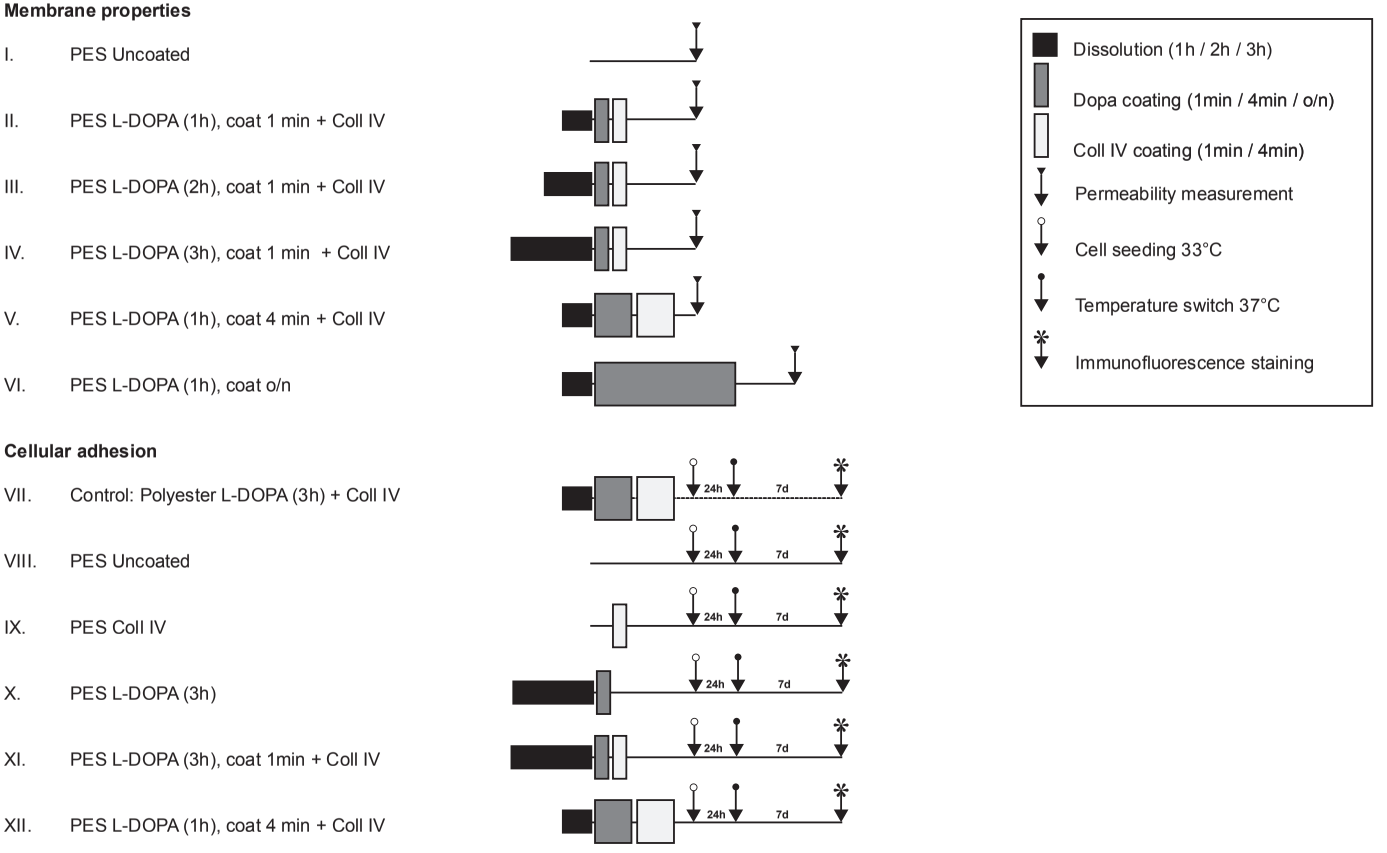

Figure 5.1 Schematic overview of the coating conditions evaluated for their membrane properties and cell adhesion.
See text for further details and abbreviations.

with the membrane surface. To obtain single sided coating of the flat PES membranes, the coating solution was first poured in glass Petri dishes and the membrane was gently placed on the top of the solutions with the porous side in contact with the liquid. The selection of final concentrations, dissolution times and coating times for both the L-DOPA and the Coll IV solution was based on cell adhesion, cell monolayer formation and tight junction expression. Reproducibility of the coating was investigated in terms of water and protein transport properties. For cell culturing, polyester Corning (Corning Costar, NY, USA) custom Transwell® supports were used. In these Transwells (12mm in diameter), the polyester cell membrane was replaced by PES - 50 membrane used for the double coating development. Here, membrane coating was performed on one side of the membrane by applying the solutions to the apical (cell-facing) side of the Transwell®. Since the PES membrane is sealed on the perimeter to the plastic holder, the basolateral side of the membrane did not come into direct contact with the coating solution. Supplemental figure A1 provides a schematic overview of a coated Transwell® membrane. As a reference, we employed double-coated (positive control) polyester Transwell® membranes, which are frequently used for *in-vitro* PTEC culture [30,33].

Membrane morphological and surface composition analysis

Membrane morphology was determined by scanning electron microscopy (FEI/Philips XL30 FEG ESEM). Surface samples were prepared and kept overnight at 30°C and gold sputtered before examination by SEM at a voltage of 5 kV. The membrane surface chemical structures and composition of the coated and uncoated PES-50 were characterized using an Attenuated total reflection-Fourier transform infrared spectra (ATR-FTIR) (Spectrum two,, Perkin Elmer, Waltham, USA) instrument. Elementary analysis of the membrane composition was performed by Energy Dispersive Spectroscopy analysis (EDS -EDAX Apollo X for ESEM, New Jersey, USA).

Membrane transport properties

Pure water, bovine serum albumin (BSA, A2153, Sigma Aldrich, Zwijndrecht, the Netherlands) and immunoglobulin G (IgG, I9640, Sigma Aldrich, Zwijndrecht, the Netherlands) solution permeance was determined using an air-pressurized dead end 'Amicon type' ultrafiltration cell (hosting 4 cm Ø membrane samples) and ultrapure water. The flux (J) of water and protein solution through the membranes at each pressure was determined by collecting the mass of the permeated liquid over time and correlating it to the membrane unit surface area. The permeance (L, in L m^{-2} h^{-1} bar^{-1}) of water or protein solution was estimated by normalizing the water or protein solution flux (J, in L m^{-2} h^{-1}) by the applied transmembrane pressure (ΔP, in bar). The permeance is, in fact, calculated from the slope of the linear part of the flux vs. transmembrane pressure relationship:

$$L = \frac{J}{\Delta P} \quad [1]$$

Clean water permeation tests were performed by using ultrapure water (MilliQ 18 MΩ.cm, Millipore; Billerica, MA, U.S.A) solutions inside stirred Amicon cells (Millipore; Billerica, MA, U.S.A), where the membranes were placed with the thin layer facing the feed solution. Automatic collection of permeate mass during time was performed by means of a Labview based software, the mass was converted to volume given the water density equal to 998 kg. m^{-3} at 20°C [34]. Uncoated flat membranes were tested after immersing them in pure water for 1 hour to remove preservatives added during the manufacturing process. Membranes double coated with L-DOPA and Coll IV were immersed in HBSS buffer for 30 min before they were used. Subsequently, the membranes were subject to a flushing step with ultrapure water at the highest working pressure (0.75 bar). Then, down to up pressure cycles were applied to each membrane.

The extent of protein transport through the membrane was evaluated by estimating the apparent sieving coefficient (SC_a).

$$SC_a = \frac{C_p}{C_b} \quad [2]$$

where C_p is the protein concentration in the permeate and C_b is the protein concentration in the feed protein solution. SC_a=1 means that the protein passes freely through the membrane, while SC_a=0 means that the membrane rejects the protein completely.

Phosphate buffered saline (PBS) containing BSA or IgG, were used at a concentration of 1 mg/ml and 0.02 mg/ml, respectively. Three different pressures (0.25, 0.50, 0.75 bar) were applied and sample collection occurred every 15 minutes for 1 hour. The protein solution was continuously mixed at the top of the membrane surface to prevent polarization concentration effect and delay membrane fouling. BSA and IgG concentration was evaluated by spectrophotometric analysis in quartz cuvettes at 280 nm.

Cell culture

A previously developed ciPTEC cell line [30] was used for functional testing of the coated membranes. In short, this immortal cell line was developed by transducing primary human proximal tubule epithelial cells, obtained from urine samples of healthy volunteers, with a SV40t, and hTERT gene. SV40t stimulates cellular proliferation at 33°C, while it is silenced by culturing the cells in a 37°C environment. As a consequence, at 37°C the cells are able differentiate and form a tight monolayer. The hTERT ensures the cellular quality, by keeping the length of the telomeres intact during proliferation. We have previously evaluated the characteristics of this cell line, on a morphological and functional level [7,31]. CiPTEC

were cultured in Dulbecco's modified eagle medium DMEM-HAM's F12 (Lonza; Basel, Switzerland) containing 10% v/v fetal calf serum (FCS) (Greiner Bio-One; Alphen a/d Rijn, the Netherlands), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 40 pg/ml tri-iodothyronine, all purchased from Sigma-Aldrich Co. (Zwijndrecht, the Netherlands). Culture media were phenol-red and antibiotic free. To prevent de-differentiation of ciPTEC during regular culturing, cells were used in experiments up to a maximum of 40 passages, during which proximal tubular characteristics remained unaltered [30]. Proliferating ciPTEC were seeded onto the polyester or PES membrane Transwell® inserts at a density of 133.000 cells/cm^2. To promote initial cellular attachment and proliferation, ciPTEC were cultured for 24 h at 33°C 5% (v/v) CO_2 and after this time period the temperature was changed to 37°C for 7 days to promote the formation of a differentiated monolayer.

Immunocytochemistry

To investigate morphological characteristics and monolayer integrity of matured ciPTEC cultured on polyester [30,33] and PES-50 Transwell® inserts, immunocytochemistry was performed and after 7 days the expression of the tight junction protein, ZO-1 (zonula occludens – 1), was studied. Matured ciPTEC were fixed using 2% (w/v) paraformaldehyde in HBSS supplemented with 2% (w/v) sucrose for 5 min and permeabilized in 0.3% (v/v) Triton X-100 in HBSS for 10 min. To prevent non-specific binding of antibodies, cells were exposed to block solution containing 2% (w/v) bovine serum albumin fraction V (Roche, Woerden, the Netherlands) and 0.1% (v/v) Tween-20 in HBSS for 30 min. Cells were incubated with antibodies diluted in block solution against the tight junction protein zonula occludens 1 (ZO-1, 1:50 dilution, Invitrogen, Carlsbad, CA) for 1 h, followed by incubation with goat-anti-rabbit-Alexa488 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, the Netherlands) for 30 min. Finally, DAPI nuclei staining (300 nM, Life Technologies Europe BV) was performed for 5 min. Protein expression and localization were examined using the Olympus FV1000 Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan) and images were captured using the Olympus software FV10-ASW version 1.7. To semi-quantify the fluorescent staining of ZO-1, a grid of 8.5 μm^2 was placed on top of the confocal images using ImageJ software (ImageJ 1.46r, NIH, USA) [35]. The number of intersections between the grid and the ZO-1 signal was determined for each condition.

Transepithelial transport measurements

Transepithelial transport of [^{14}C]-creatinine by matured ciPTEC monolayers cultured on either polyester or PES Transwell® inserts was measured using a Transwell® culture system. A schematic overview of the set-up, is provided in the supplemental file (Figure 5.A1). Polyester Transwells® (diameter 12mm, pore size 0,4μm) were coated with L-Dopa alone or in combination with Coll IV (25 mg/ml, for 2 hours), and compared to double coated polyester

or PES-50 Transwells®. Mature cell monolayers were washed in modified Krebs-Henseleit buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands) including 10mM Hepes (pH 7.4). Subsequently, all membranes tested were pre-incubated for 2 h at 37°C, 5% (v/v) CO2 in Krebs-Henseleit-Hepes buffer (0.5 mL apically, 1.5 mL basolaterally). Transport was initiated by the basolateral addition of either [^{14}C]-creatinine (0.75 μM, 2 μCi/ml) or [^{3} H]-inulin (0.45 μM, 20 μCi/ml) with or without cimetidine (100 μM) or metformin (100 μM) as competitors for OCT2 transport (Sigma-Aldrich Co., Zwijndrecht, the Netherlands). At the start of the measurement, a 20 μL reference sample was taken from the basolateral exposure compartment. After 30 min of incubation with gentle agitation at 37°C a 200 μL sample was removed from the apical chamber. The activity of [^{3}H] and [^{14}C] in the samples was determined by liquid scintillation counting (Beckman). Fluxes of [^{14}C]-creatinine or [^{3}H]-inulin were determined for each separate Transwell®. The flux of [^{3}H]-inulin was used as an internal leakage marker. The basolateral to apical flux (J) was calculated with:

$$J = \frac{dQ}{S * dt} \qquad [3]$$

where dQ = amount transported [pmol]; dt = duration of transport [minutes]; S = surface area [cm^2].

Data analysis

Water, BSA and IgG transport measurements in each experiment were performed at least in triplicates (N≥3 for each coating condition). Results are presented as mean ± standard deviation. Statistical analysis was performed with the Microsoft Excel® software (Microsoft Corporation, Seattle, USA) using a one-way ANOVA or a Student's T-test when appropriate.

Quantification of ZO-1 staining on Transwell® polyester or PES membranes was performed in triplicates for each coating condition. The results are presented as mean ± sem. Results were normalized by taking the double coated Polyester Transwell® as 100% reference. One-way ANOVA followed by a Dunnett's post-test, was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA).

For basolateral creatinine transport experiments in the commercially available polyester Transwells® the experiments were performed at least in triplicate in three independent experiments. The custom PES Transwells® were tested in duplicate in three independent experiments. Results are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism, using a two way ANOVA combined with a Bonferroni's post-test.

RESULTS – DISCUSSION

The biofunctionalization strategy of flat PES-50 membranes was optimized first to develop functional ciPTEC monolayers. Artificial membranes should provide a solid base for cells to form tight monolayers and facilitate active transepithelial solute transport. In the nephron, the proximal tubule is responsible for the reabsorption of 65% of all the water filtered by the glomerulus; the very high permeability of the tissue was estimated to be about $1x10^5$ L m^{-2} h^{-1} bar^{-1} [36]. The hydraulic permeability of native PES-50 membrane is 2 orders of magnitude lower than the value reported for natural tissue, the membrane was chosen on the base of its capacity to retain vital blood components, consistent with other research in the field [22]. For this reason, water transport through the coated membranes was one of the main parameters to optimize, in order to preserve the highest permeance possible.

A recent study proposed the application of an overnight double coating [10]. However, when we applied such a coating, a significant decrease in membrane water permeance, (39 ± 23 L m^{-2} h^{-1} bar^{-1}) was detected in comparison to uncoated membranes (738 ± 110 L m^{-2} h^{-1} bar^{-1}). Further reduction of the permeance was expected when applying the second coating layer consisting of Coll IV. To avoid significant transport limitations, we investigated shorter L-DOPA dissolution times. In a first attempt, L-DOPA was used for the coating immediately after the dissolution step (L-DOPA (1 h)). The average value of water permeance did not change significantly (654 ± 70 L m^{-2} h^{-1} bar^{-1}) with respect to uncoated membrane values (Figure 5.1, I). After applying the Coll IV coating on top of the L-DOPA layer (Figure 5.1, II), the average value of water permeance decreased to 347± 151 L m^{-2} h^{-1} bar^{-1}. However, the high coefficient of variation (0.44) indicated a non-reproducible coating. These results indicate that short coating times do not cause significant changes, whilst overnight coating results in a dramatic reduction in membrane transport properties. Since the variable "time" plays a central role in the macroscopic transport properties of the coated membrane, we compared the effects of prolongation of the L-DOPA dissolution time (Figure 5.1; II, III and IV), to prolongation of the coating time to L-DOPA and Coll IV from 1 to 4 min (Figure 5.1; II, V).

When applying L-DOPA coating alone, prolongation of the L-DOPA dissolution time slightly increases the average membrane water permeance, although this effect was not significantly different from the non-coated PES-50 membranes (dark symbols, Figure 5.2a). Literature reports that L- DOPA coating can increase the membrane hydrophilicity due to the coexistence of carboxylic and amino groups in the L- DOPA molecule [37]. In fact, the effect of L-DOPA coating on hydrophilization of different materials has been shown by the reduction of water contact angle under the same experimental conditions used in the present study (pH, Tris buffer molarity and L-DOPA concentration) [21,37,38] we therefore expect that our membranes are slightly hydrophilized, too. Nevertheless, the application of L-DOPA to porous membranes can reduce the membrane transport properties. This decrease mostly occurs to UF membranes where the pore dimensions allow migration of L-DOPA monomers

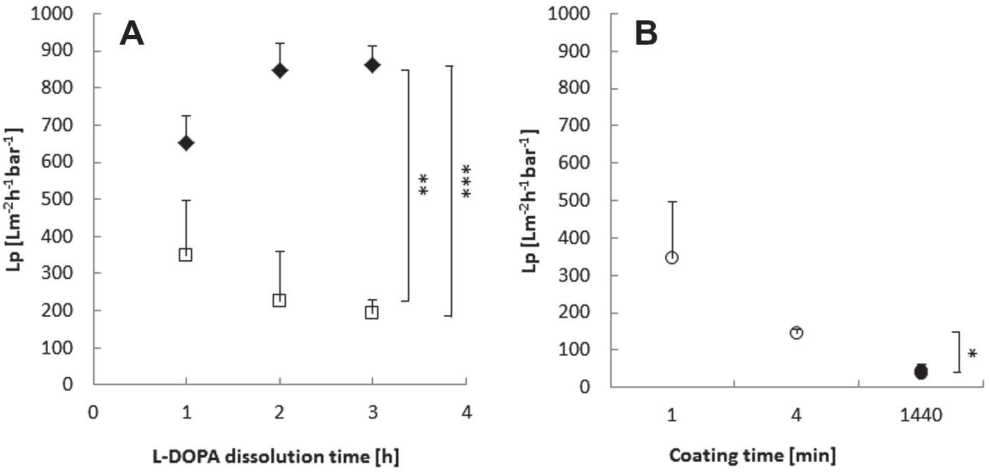

Figure 5.2 Pure water permeance of double coated PES-50 membranes.
Pure water permeance of PES-50 membranes coated with L-DOPA (2 mg/ml) and Coll IV (25 µg/ml) as a function of: a) L-DOPA dissolution time for PES membranes coated for 1 min with L-DOPA solution only (◆), or L-DOPA followed by Coll IV (□); b) double coating with L-DOPA followed by Coll IV with varying coating times (○), or L-DOPA solution after overnight coating (●). Data are presented as mean ± standard error, * $p<0.05$; ** $p<0.01$; *** $p<0.005$; N≥3 for each experimental point.

and oligomers inside the membrane structure with consequent reduction or blockage of the inner porosity [39,40]. The transport properties of reverse osmosis (RO) and microfiltration (MF) membranes was reported to be less affected by L-DOPA coatings, due to the different pore size [40]. Since we used PES-50 UF membranes, the coating time as well as the L-DOPA dissolution time required optimization to avoid complete suppression of the membrane permeance.

The Coll IV coating on top of the L-DOPA layer (Figure 5.1, conditions I – IV) decreased the membrane water permeance significantly (open symbols in Figure 5.2a). The permeance for the double coated membranes with L-DOPA (3 h) and Coll IV for 1 min was 194 ± 35 L m^{-2} h^{-1} bar^{-1}, which is significantly lower than the uncoated membranes. However, when compared to overnight L-DOPA coating (Figure 5.1; VI), the membrane permeance improved markedly. Furthermore, prolongation of L-DOPA dissolution to 3 h enhanced the coating reproducibility, as demonstrated by the low standard deviation of the permeance values. Figure 5.2b presents the optimization procedure of the double coating using different coating times of L-DOPA (1 h) (Figure 5.1; II, V and VI). The water permeance for double-coated membranes with 4 min coating was A 2.7 fold higher (146 ± 10 L m^{-2} h^{-1} bar^{-1}) than for overnight L-DOPA (only) coating. Prolongation of coating time to 4 min also improved the double coating reproducibility, as demonstrated by the low standard deviation of the permeance results. These results indicate that optimization of the L-DOPA dissolution, and coating

times significantly improves the membrane permeance and coating reproducibility, when compared to overnight coating.

The progressive reduction of membrane water permeance correlated with the observed morphological changes at the porous side of the membrane surface where the double coating was applied. Figure 5.3 presents SEM images of uncoated, only L-DOPA and double coated membranes surfaces. The single L-DOPA coating slightly affected the porous layer morphology (data not shown). After application of L-DOPA (3 h), an estimated 40% reduction in the macroporosity visible at the upper surface of the flat membranes could be observed compared with the original membranes (Figure 5.3b). Application of Coll IV to the L-DOPA coating resulted in complete occlusion of membrane surface pores as compared to membranes coated with L-DOPA only, for all L-DOPA dissolution times tested (Figure 5.3c). The lack of visible differences in surface morphology in the presence of the single L-DOPA coating in comparison with native membranes was consistent with the average rate of PDA layer deposition (2 nm/h) reported in previous studies for the same experimental conditions (L-DOPA concentration, temperature, pH and Tris buffer molarity) [21,41]. The analysis of membrane surface chemical composition confirmed that the thickness of the coating was in the nm range, as the beam penetration for both EDAX and FT-IR analysis was deeper than the single or double coating applied. In fact for both the FT-IR spectrum and the elementary composition, the underlying PES material is still dominant (Figure 5.4). Dopamine monomers and small oligomers have previously been demonstrated to play a primary role in the initiation of the PDA deposition and its consequent polymerization at the membrane surface [21,40,41]. In these studies, maximal monomer deposition rate was observed during the first hours after L-DOPA dissolution. This finding may explain why, in our case, a short coating time was sufficient to modify the PES-50 membranes, providing good collagen IV adhesion and reproducibility of transport properties.

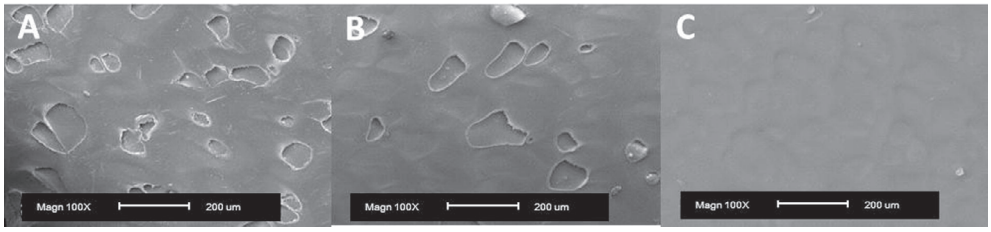

Figure 5.3 Scanning Electron Microscopy analysis of (un)coated PES-50 membranes.
Representative Scanning Electron Microscopy analysis of PES-50 membranes porous side surface morphology with L-DOPA (2 mg/ml) and Coll IV (25 µg/ml) with different L-DOPA dissolution times: a) uncoated; b) 1 min coating with L-DOPA (3h) only; c) 4 min coating with L-DOPA (1h) and Coll IV. Magnification 100X. At least three independent membranes were examined for each condition.

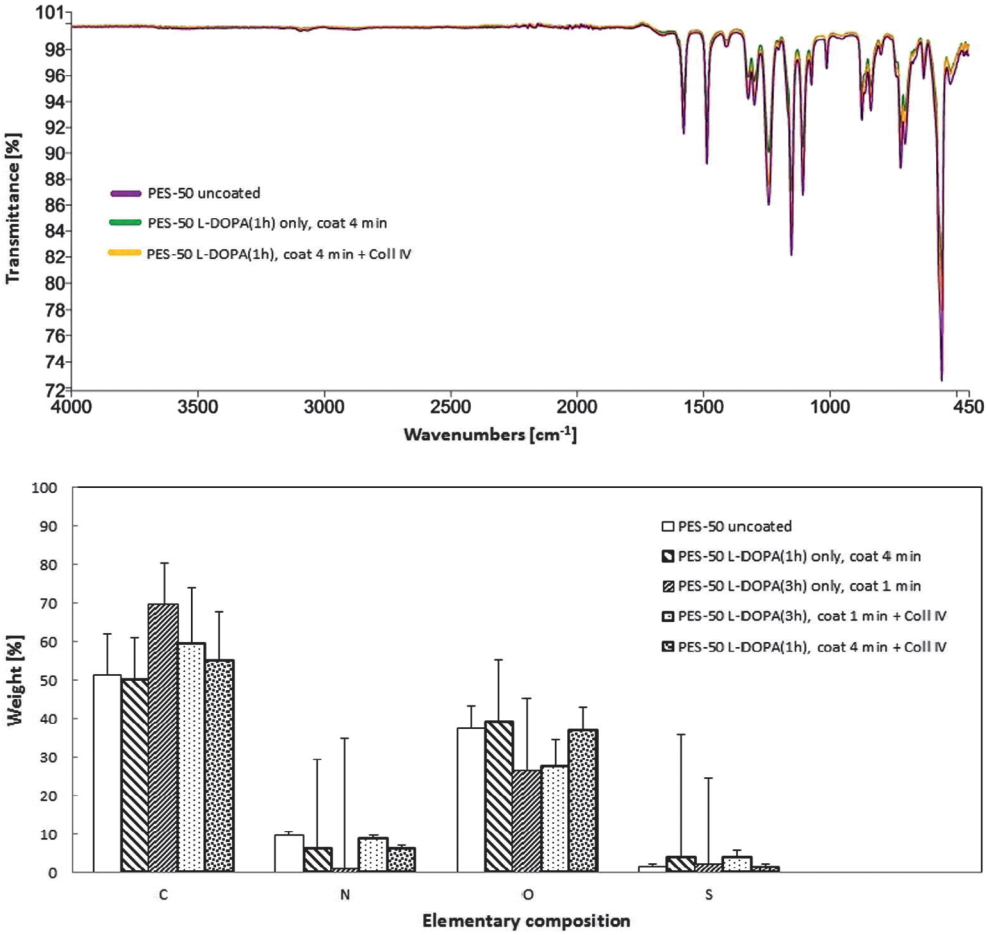

Figure 5.4 Surface chemical composition of PES-50 membranes uncoated and coated with L-DOPA (2 mg/ml) and Coll IV (25 µg/ml).
Above ATR-FTIR spectra for: purple) uncoated PES-50; green) 4 min coating with L-DOPA (1h) only; yellow) 4 min coating with L-DOPA (1h) and Coll IV. Below EDAX analysis of (from left to right: uncoated PES-50; 4 min coating with L-DOPA (1h) only; 1 min coating with L-DOPA (3h) only; 1 min coating with L-DOPA (3h) and Coll IV; 4 min coating with L-DOPA (1h) and Coll IV.

The optimized coatings were tested further for optimal cell monolayer formation by ZO-1 tight junction protein expression, a marker that indicates monolayer integrity and polarity of epithelial cells. The tested coating conditions are described schematically in Figure 5.1, under the cellular adhesion heading (Figure 5.1; conditions VII-XII). Standard double coated polyester Transwell® inserts, were used as a positive control (Figure 5.5a). Uncoated PES-50 membranes provided poor cell adhesion of ciPTEC (Figure 5.5b) while single coating with Coll IV (25mg/ml) or L-DOPA (2mg/ml) did not improve this (Figure 5.5c,d). After application of the double coating, cellular adhesion and the expression of ZO-1 improved

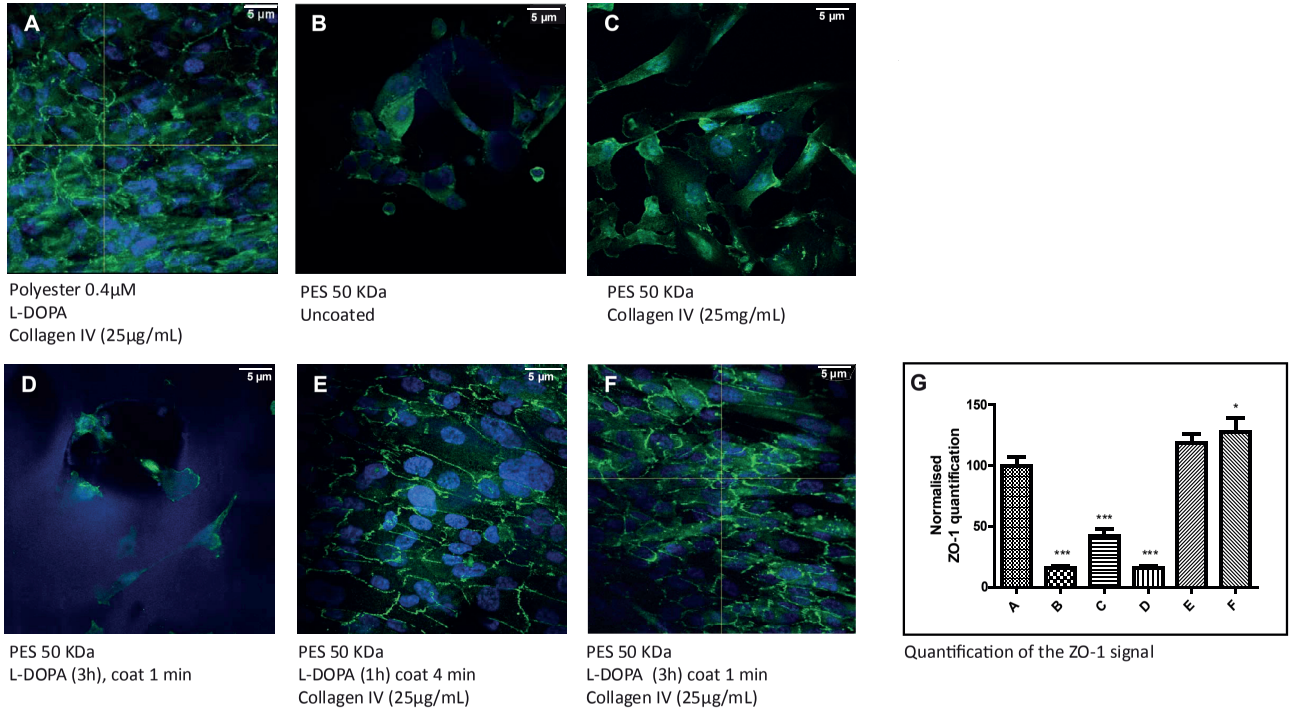

Figure 5.5 Representative images of immunocytochemical analysis of the ZO-1 tight junction protein (green) and nuclei (blue) in ciPTEC monolayers cultured on membranes with varying coating times.

a) L-DOPA (2 mg/ml) and Coll IV (25 µg/ml) double coated Polyester Transwell® inserts; b) uncoated PES - 50 Transwell® insert; c) Coll IV (25 µg/ml) coated PES-50 Transwell® inserts d) L-DOPA (2 mg/ml) coated PES - 50 insert; e) PES-50 Transwell® inserts coated for 4 min with L-DOPA (1h) and Coll IV; f) PES-50 Transwell® inserts coated for 1 min with L-DOPA (3h) and Coll IV. G) ZO-1 abundance in ciPTEC monolayers compared to double coated Polyester Transwell® inserts. At least three independent membranes were examined for each condition. ***p<0.005 Magnification 60x.

(Figure 5.5e-f). The observed clear ZO-1 expression indicates polarization of the monolayer, improving epithelial characteristics [42] and limits paracellular leakage. No significant difference in monolayer formation and ZO-1 expression was detected between conditions XI and XII (Figure 5.5e,f), which varied either the coating or dissolution time. Furthermore, quantification of the ZO-1 has indicated that ciPTEC expressed ZO-1 protein in a similar fashion on both double coated PES-50 as on polyester Transwells®, which are considered the golden standard in ciPTEC transepithelial studies (Figure 5.5g). Lowering the concentration of L-DOPA reduced the reproducibility of cellular adhesion, even in the presence of a higher Coll IV concentration, thus suggesting the importance of a good L-DOPA layer to foster Coll IV membrane coating. Furthermore, increasing the Coll IV concentration to 50 µg/ml after 2 mg/ml L-DOPA coating did not improve monolayer quality with respect to 25 µg/ml Coll IV (Figure 5.A2, supplemental Figure A2).

The coatings were investigated further in terms of protein rejection and fouling phenomena during protein solution permeance. Table 1 shows a comparison of transport properties of uncoated and membranes with optimized coatings (Figure 5.1; conditions I, IV and V). The permeance of IgG and BSA solutions for the uncoated PES-50 membranes is 53 ± 11 and 57 ± 7 L m^{-2} h^{-1} bar^{-1}, respectively, about 96% lower than the water permeance through the same membranes (P<0.05). There was no significant difference in protein solution permeance for the coated membranes, as values ranged from 37 ± 27 L m^{-2} h^{-1} bar^{-1} to 65 ± 24 L m^{-2} h^{-1} bar^{-1}. Both BSA (66 kDa) and IgG (150 kDa) were almost completely rejected by both uncoated and coated membranes: the SC < 0.1, consistently to what is expected for a 50 kDa membrane.

Table 1 Comparison of the transport properties of uncoated PES-50 membrane and coated with L-DOPA (2 mg/ml) and Coll IV (25 µg/ml) with two approaches: 1) 1 min coating with L-DOPA (3h) and 1 min Coll IV; 2) 4 min coating with L-DOPA (1h) and 4 min Coll IV.

		Uncoated	1 min L-DOPA (3 h) + 1 min Coll IV	4 min L-DOPA (1 h) + 4 min Coll IV
Pure water permeance	[L m^{-2} h^{-1} bar^{-1}]	738 ± 110	194 ± 35	146 ± 10
BSA solution permeance	[L m^{-2} h^{-1} bar^{-1}]	57 ± 7	44 ± 8	37 ± 27
IgG solution permeance	[L m^{-2} h^{-1} bar^{-1}]	53 ± 11	65 ± 24	51 ± 12
BSA $SC_a = C_P/C_b$	[–]	0.03 ± 0.01	0.06 ± 0.02	0.02 ± 0.01
IgG $SC_a = C_P/C_b$	[–]	0.02 ± 0.01	0.05 ± 0.03	0.04 ± 0.03

Given the similarity between the transport properties and the similar cell behavior for the membranes with the optimized coatings, the transport of inulin and creatinine was measured on membranes coated with the coating which required the shortest experimental time: L-DOPA (1h) with a coating time of 4 min for both L-DOPA and Coll IV (Figure 5.1, XII). Transepithelial transport of [^{14}C]-creatinine was measured in presence and absence of

transporters substrates, acting as competitors to inhibit uptake. In the kidney, approximately 20% of creatinine is actively transported by organic cation transport proteins [43]. The functionality of the basolateral cation uptake by organic cation transporters (OCTs) in ciPTEC has previously been demonstrated [7]. Here, Figure 5.6 presents the transmembrane flux of [^{14}C]-creatinine (0.75 μM, Figure 5.6 a, b, c) and [^{3}H]-inulin (0.45 μM, Figure 5.6 d, e, f) by ciPTEC cell monolayers, cultured on double coated PES-50, polyester Transwell® membranes or Coll IV coated polyester Transwell® membranes. The paracellular leakage of [^{3}H]-inulin across the monolayer was used as an indicator of passive diffusion, hence, monolayer tightness, since active transcellular transport has not been reported for this compound [44]. In all cases, the inulin leakage was very low, indicating that a tight monolayer was achieved. The observed average leakage of 0.8 ± 0.1 pmol min^{-1} cm^{-2} (0.1% of the total inulin amount) is comparable to observations made in previous studies that utilized MDCK or LCC-PK cells [45]. In accordance, the organic cation transport inhibitors, metformin and cimetidine, did not affect inulin transport across the cell monolayers. The creatinine flux through the double-coated "living" PES-50 membranes (2.8 ± 0.1 pmol min^{-1} cm^{-2}) was 3.5 times higher than the inulin flux (0.8 ± 0.03 pmol min^{-1} cm^{-2}). Importantly, the creatinine flux was significantly decreased by the inhibitors, metformin (24 ± 6% decrease, P<0.05) and cimetidine (18 ± 0.2% decrease, P<0.05), indicating that the developed "living" membrane actively secretes creatinine. The results of the "living" PES-50 membranes were comparable to those obtained for cells cultured on the golden standard Polyester - Coll IV Transwell® system (see Figure 5.6 and supplemental Figure A3 which compare the permeance of inulin and creatinine through the various membranes).

Various studies have used cellular over-expression models to demonstrate transepithelial creatinine transport. We currently show this trans-epithelial secretory pathway is endogenously present and functional in ciPTEC. Limited data are available for creatinine fluxes in human renal cells. Brown et al. 2008 reported transepithelial creatinine fluxes for primary human proximal tubule epithelial cells [46]. Although we observed lower creatinine fluxes in ciPTEC, the transport capacity is maintained for up to 40 passages (unpublished data). This underscores the robustness of this model and its potential application in a RAD. The use of an immortal cell line provides unlimited proliferative capacity, providing the option to expand the surface area of the living membrane necessary to reach the required clearance levels.

Previous studies have shown that the proximal tubule cell can take up free L-DOPA through the organic cation transport system, thereby influencing cellular processes [47,48]. However, since the L-DOPA coating was fully polymerized before application of Coll IV, and several additional pre-culture washing steps had been applied, no free L-DOPA is expected to be present when culturing the cells. This is supported by the finding that no detrimental effect of the coating was observed for either transepithelial ^{14}C-creatinine transport or for the morphology of ciPTEC.

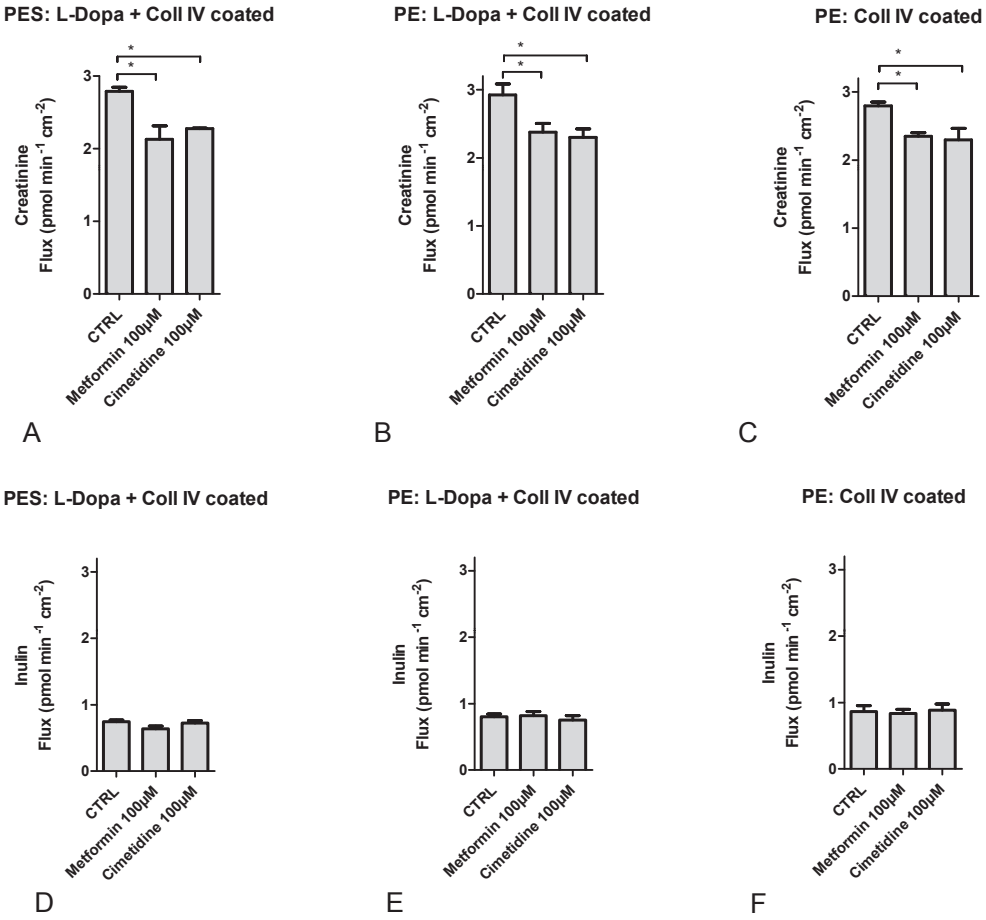

Figure 5.6 Basolateral to apical creatinine and inulin fluxes.
Basolateral to apical fluxes of (a,b,c) [^{14}C]-creatinine (0.75 µM) and (d,e,f) [$^{3\,h}$]-inulin (0.45 µM) across ciPTEC monolayers. Measurements were performed in the presence or absence of the inhibitors metformin (100 µM) or cimetidine (100 µM). Depicted are ciPTEC monolayers cultured on: (a,d) PES-50 Transwell® inserts coated with L-DOPA(1h) and Coll IV; (b,e) Polyester Transwell® membranes coated with L-DOPA(1h) and Coll IV; (c,f) Polyester Transwell® membranes coated with Coll IV only. All coatings were applied for 4 minutes at the following concentration: L-Dopa 2mg/ml, Coll IV 25mg/mL. Data are presented as mean ± standard error, N=3 *P<0.05

Although we focused here on creatinine transport as a marker for endogenous secretion processes in ciPTEC, creatinine is not the only substance that has to be cleared from the blood. Many cationic and anionic waste products, including indoxyl sulfate, hippuric-acid, kynurenic acid, polyamines and guanidines, are retained in patients suffering from uremia [49]. Previously, we reported that ciPTEC functionally express various membrane transport proteins that are involved in the uptake and excretion of uremic toxins (UTs) [7,30,50]. There-

fore a device based on cultured monolayers of ciPTEC grown on modified PES-50 surfaces could provide the ideal tool in the removal of these compounds. The use of sophisticated bioanalysis techniques such as LC-MS/MS might provide more opportunities for the transepithelial transport measurements of these toxic compounds by ciPTEC monolayers [51]. As a next step in RAD development, long term stability and preservation of functionality in ciPTEC monolayers should be evaluated. Furthermore, the introduction of dynamic culture conditions could improve both the formation of tight junctions and cellular transport properties [52]. Recent work suggests beneficial effects of shear stress on renal epithelia, leading to increased endocytosis [45] and sodium reabsorption by the NHE3 receptor [53,54]. Evaluation of the shear stress on the functionality of a transporting ciPTEC monolayer would provide valuable information for the development of a bioartificial device.

CONCLUSIONS - OUTLOOK

In this study, we showed the proof of concept for creating a "living membrane" by combining functional ciPTEC with PES-50 membranes coated with L-DOPA and Coll IV. The coating was optimized to achieve retention of vital blood components whilst preserving high water permeance and optimal cell monolayer formation. The transport of creatinine through the developed "living membrane" was comparable to golden standard systems for studying transepithelial transport across proximal tubule monolayers *in-vitro*. The results reported here are promising for the development of a bioartificial kidney. Further investigations are required to establish the long-term stability of these functional renal cell monolayers. Future studies will be directed towards producing "living membranes" on hollow fiber PES-based membranes and to evaluate up-scaling in a bioreactor system to develop a clinically relevant device.

ACKNOWLEDGMENT

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SUPPLEMENTARY DATA

Supplementary data is available at http://www.ncbi.nlm.nih.gov/pubmed/25527093

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6

Dynamic cell culture conditions improve transepithelial organic cation transport by human conditionally immortalized proximal tubule epithelial cells

Carolien M.S. Schophuizen[1], Joost G.J. Hoenderop[2], Lambert P. van den Heuvel[1,4*] and Rosalinde Masereeuw[3*],

* Authors contributed equally

Departments of [1]Pediatric Nephrology, [2]Physiology, and [3]Pharmacology and Toxicology, Radboudumc, Nijmegen, The Netherlands.
[4]Department of Development and Regeneration, Catholic University Leuven, 3000 Leuven, Belgium

ABSTRACT

Proximal tubule epithelial cells are commonly used as a model to investigate renal toxicity, drug-drug interactions, or to examine renal clearance processes. Previously, we developed a conditionally immortalized proximal tubule epithelial cell line (ciPTEC) that enables studying active transepithelial transport of organic cationic solutes under static culture conditions. Here, we evaluated the effects of fluid flow by shear stress on the cell monolayer quality, as well as the transepithelial creatinine transport capacity of the ciPTEC monolayer.

To simulate fluid flow, confluent ciPTEC monolayers were cultured for seven days under dynamic conditions on a 2D plate rocker. Dynamic cell culture conditions increased cilia length significantly (6.9 ± 0.9 μm), compared to statically cultured cells (5.7 ± 0.4 μm, p=0.01). Monolayer height increased from 9.7 ± 4.8 μm to 15.5 ± 5.2 μm (p<0.005). The expression of various organic cation transporters was induced, leading to increased cellular ASP^+ uptake (until 151 ± 28%, p<0.05) Furthermore, the transepithelial creatinine flux in dynamically cultured cells cultured was 1.4 x higher than the flux in statically cultured cells (2.9 ± 0.4 pmol min^{-1} cm^{-2} vs. 2.1 ± 0.4 pmol min^{-1} cm^{-2}, respectively; p<0.001). When cells cultured on a 2D rocker were exposed to a mixture of cationic uremic toxins (UTs), the viability decreased significantly indicating higher cellular sensitivity (IC_{50} static: 27.9 ± 2.7 fold the uremic plasma concentration reported in literature vs. IC_{50} shaking: 16.8 ± 2.3 fold the uremic concentration of the UTmix, p<0.01).

In conclusion, culturing ciPTEC under shear stress conditions on a 2D plate rocker increased the mRNA expression of various transport proteins, stimulated transepithelial organic cation transport, and enhanced sensitivity to cationic UTs. These findings strongly suggest that the introduction ciPTEC to apical shear-stress can stimulate further differentiation of this cell type.

INTRODUCTION

Renal proximal tubule epithelial cells (PTEC) are routinely used to study cellular clearance processes, drug-drug interactions or renal toxicity. To this end, a wide variety of epithelial cell models are being cultured on plastic culture surfaces, or specialized membranes that allow the formation of an apical and basolateral compartment [1]. PTEC are highly polarized, have a distinct basolateral to apical orientation, and express a specific set of solute transporters on either side. The proximal tubular transport proteins facilitate the clearance of large, charged or protein bound (retention) solutes from the circulation into the pro-urine [2]. As these solutes can freely pass the glomerulus, active secretion in the renal tubule is necessary for their removal. Although renal replacement therapy, such as hemodialysis, can prolong the life expectancy of patients suffering from end-stage renal disease, the clearance of uremic retention solutes is inefficient and systemic accumulation can promote the development of secondary morbidities [3,4].

Our previous work has shown that a human conditionally immortalized tubule epithelial cells (ciPTEC) endogenously express functional organic cation transporter 2 (OCT2, *SLC22A2*) in concert with other essential organic cation transporters, such as OCT1, OCT3, p-glycoprotein (P-gP, *ABCB1*), breast cancer resistance protein (BCRP, *ABCG2*) and multidrug and toxic compound extrusion MATE proteins (*SLC47*) [5,6]. These transporters can facilitate the clearance of drugs and waste products, such as creatinine and specific cationic uremic toxins (UTs) [7-9]. Furthermore, ciPTEC form functional cell monolayers on polyethersulfone based dialysis membranes. An optimized coating comprising both L-DOPA and collagen type IV promoted cell monolayer formation, while facilitating organic cationic transepithelial transport [8]. Current research actively explores the possibilities to utilize the functional properties of cultured renal epithelial cells in an add-on device to renal hemodialysis therapy. Such a bioartificial kidney, or so-called renal assist device (RAD), aims to remove uremic retention solutes in dialysis patients more efficiently. However, a RAD would require cellular monolayers to function within a dynamic system, characterized by fluid flow. While ciPTEC were shown to actively transport cationic compounds when cultured under static conditions, thus far the effects of fluid flow on ciPTEC solute transport has not been elucidated.

Over the last 30 years, the effect of flow on cultured cells has steadily gained interest. Numerous studies have investigated the effect of flow induced shear stress on vascular endothelial cells [10]. The vascular shear is estimated between 20 and 40 Dyn/cm^2 and has been reported to regulate many processes, such as angiogenesis, release of vasoactive substances, permeability and the expression of adhesion molecules [11-13]. In contrast, fluid flow in the renal tubule is expected to be much lower and mainly dependent on the single nephron glomerular filtration rate [14,15]. A number of studies have evaluated the *in-vitro* effect of fluid flow on renal proximal tubule epithelium, by making use of innovative flow chamber devices. Duan

et al. reported flow induced rearrangement of the cytoskeleton, increased the Na^+ and HCO_3^- absorption, and enhanced basolateral Na^+/K^+-ATPase expression [16,17]. Furthermore, Jang *et al.* described a dual chamber microfluidic device in which the proximal tubular epithelium was exposed to apical flow, leading to enhanced cellular albumin and glucose reabsorption, alkaline phosphatase activity and substrate efflux through the P-glycoprotein (P-gp) transporter [18].

The effects of fluid flow observed in these studies prompted us to investigate the effects of flow on ciPTEC organic cation transport. To simulate fluid-flow in our cell model, cells were cultured under dynamic conditions on a 2D plate rocker. Such a simple system provides the possibility to use standard or Transwell® cell culture dishes, and the use of small amounts of culture medium avoiding dilution of compounds transported [19]. Here, we applied the shear-stress to ciPTEC for up to 7 days. We examined the effects of the dynamic culture conditions on the monolayer quality, and evaluated the transepithelial creatinine transport capacity of the ciPTEC cell model.

METHODS

Cell culture

Experiments were performed using the previously developed, immortalized and well characterized human ciPTEC, obtained from a healthy volunteer's urine sample [5]. CiPTEC were routinely cultured in Dulbecco's modified eagle medium DMEM-HAM's F12 (Lonza; Basel, Switzerland) containing 10% v/v fetal calf serum (FCS) (Greiner Bio-One; Alphen a/d Rijn, the Netherlands), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor and 40 pg/ml tri-iodothyronine, all purchased from Sigma-Aldrich Co. (Zwijndrecht, the Netherlands). Culture media were phenol-red and antibiotic free. To prevent dedifferentiation of ciPTEC during regular culturing, cells were used up to a maximum of 40 passages, during which proximal tubular characteristics remained unaltered [5]. For experiments performed on regular plastic cell culture surfaces, cells were seeded at 30% density and left to attach for 24 h at 33° C. When seeded on 12 well polyester Transwell® permeable supports (Corning Costar, NY, USA), membranes were first treated with a collagen IV coating as described previously [8], to promote monolayer formation. Collagen IV (25 μg/mL) solution was applied to the membrane surface for 4 min. After aspiration and drying (5 min), the membranes were washed three times in HBSS to remove any remaining solvent or unbound compound. Proliferating ciPTEC were seeded onto polyester Transwell® membrane inserts at a density of 133.000 cells/cm^2. Routine culture was performed at 33°C, 5% (v/v) CO_2, media was refreshed every 2 or 3 days. Subsequently, the temperature was changed to 37°C to induce monolayer maturation for 7 days prior to performing the experiments.

Dynamic culture conditions

An UltraCruz™ 2D rocker (Heidelberg, Germany) with a tilt angle of 13° was used to generate flow at a range of 30 oscillations per min. Cell layers cultured under shaking conditions were seeded as stated above. After the initial 24 h attachment period, the temperature was changed to 37°C and at the same time the cells were transferred to the 2D rocker for 7 days of maturation under so called dynamic culture conditions. The characteristic fluid shear stress (the shear stress at the center of dish bottom when the dish is horizontal) in the round culture dishes, was quantified using the formula describing detailed analysis for the circular rocking dish presented in the supplementary material of in a publication by Zhou et al. 2010 [19]. In the case of the plastic 12 well plates (well diameter: 22.1 mm, fluid height: 2.6 mm) the maximal flip angle was set at the critical flip angle of 0.23 Rad, rotating at 1 cycle/sec, leading to a characteristic shear stress of 0.056 Pa or 0.56 Dyn/cm^2. For the Transwell® membranes (well diameter: 13 mm, fluid height: 0.44 mm), the maximal flip angle was also set at 0.23 Rad. Note that 0.23 Rad was the maximal angle for which the basolateral compartment retained fluid contact. The shaker was set to rotate at 1 cycle/sec, leading to a characteristic shear stress of 0.026 Pa or 0.26 Dyn/cm^2. As a control, cells were cultured under static conditions in the same incubator.

Immunocytochemistry

Cells cultured on Transwell® membranes were washed twice with pre-warmed Hank's Balanced Salt Solution (HBSS) (Gibco, Life, Technologies, Bleiswijk, the Netherlands). Then the ciPTEC monolayers were fixed for 5 min at room temperature using a fixation solution containing 2% (w/v) Paraformaldehyde, 4% (w/v) sucrose in HBSS. Subsequently the cell monolayers were washed twice in wash buffer; 4% (w/v) FCS, in HBSS. The cell layer was permeabilized using HBSS containing 0.3% (w/v) Triton, and again washed in wash buffer for 5 min. After blocking the samples for 30 min in block solution (HBSS containing 2% (v/v) FCS, 4% (w/v) BSA and 0.1% (w/v) Tween20), the samples were incubated overnight at 4°C with Acetyl-α-Tubulin antibody 1:500 (Acetyl-α-Tubulin (Lys40) (D20G3) XP® Rabbit, Cell Signaling, BioKe, Leiden, The Netherlands) added apical in block solution. The next day, after three subsequent washing steps (10 min), the filters with the cell monolayers were cut out of the Transwell® insert. The second antibody goat anti-rabbit Alexa 568 (Life Technologies, Bleiswijk, The Netherlands) was diluted 1:200 in block solution, and incubated for 30 min with 30 µL per membrane. For double staining with either the tight junction protein ZO-1 or RPGRIP1L the RPGR interacting basal body protein (affinity purified guinea pig-anti-RPGRIP1L (SNC040)) previously described by Arts et al. [20]. Membranes were incubated for at least 60 min at room temperature, with this first antibody diluted to 1:50 or 1:500 respectively. The second antibody Goat anti Rat Alexa 488 (FITC) or goat anti guinea pig were added at a 1:200 dilution (Life Technologies, Bleiswijk, The Netherlands). Subsequently, the membranes were washed and the nuclei stained with DAPI 1:1000 in block solution (Molecular Probes,

Life Technologies, Bleiswijk, The Netherlands). After a final washing step, the membranes were mounted on slides with the monolayer facing up, and covered with a cover glass. The immunofluorescence was imaged using the Olympus FV1000 Confocal Laser Scanning Microscope (Olympus, Hamburg, Germany). Monolayer height was calculated after imaging the cell monolayer in a z-stack, by subtracting the initial height at the level of the polyester membrane from the endpoint, i.e. the final image at which the immunofluorescent signal was still detectable. Cilia length was quantified by determining the distance between the RPGRIP1L signal and the ciliary tip, for at least 5 independent z-stack images per condition. The cilia length was calculated using the neurite tracer option from the segmentation plug-in present in the ImageJ software (ImageJ 1.46r, NIH, USA).

Transepithelial resistance determination

Transepithelial resistance of matured ciPTEC monolayers on Transwell® inserts was measured using the Millicell electrical Resistance volt-ohm system (Millipore, Billerca, USA). Measurements were performed as described in the manufacturer's protocol.

qPCR

Total RNA was isolated using TRIzol (Life, Technologies, Bleiswijk, the Netherlands) and chloroform extraction according to the manufacturers' protocol. 2 µg of total RNA served as a template for single-strand cDNA synthesis in a reaction using oligo (dT) and random primers in a M-MLV reverse transcriptase reaction mixture (Catalog #28025-013, Invitrogen, Bleiswijk, The Netherlands) according to the manufacturers' protocol (Doc. Rev: 100702). The mRNA expression levels were detected using gene specific primer-probe sets MATE1 (SLC47A1, hs00217320_m1), MATE2k (SLC47A2, hs00945650_m1), OCT1 (SLC22A1, Hs00427552_m1), OCT2 (SLC22A2, hs01010723_m1), OCT3 (SLC22A3, Hs01009568_m1), OATP4C1 (SLCO4C1, hs00698884_m1) and TaqMan Universal PCR Master Mix (all obtained from Applied Biosystems, CA, USA). The CFX96 Real Time PCR system (Bio-Rad Laboratories, Veenendaal, The Netherlands) was used to perform the qPCR reactions and data was analyzed using the CFX Manager™ software (Bio-Rad Laboratories, Veenendaal, The Netherlands). The reference gene GAPDH was used to normalize the mRNA expression levels. Data are expressed as fold increase ± standard range compared to ciPTEC cultured under static conditions.

ASP^+ uptake assay

ASP^+ uptake by ciPTEC was measured as described before [9]. In short, OCT activity was measured in freshly harvested matured cell suspensions by addition of 100 µM of the fluorescent OCT substrate ASP^+ in HEPES-TRIS (Hepes-Tris (10 mM), NaCl (132 mM), KCl (4.2 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), D-glucose (5.5 mM), pH was set to 7.4 using 1.5 M Tris in MQ, 37°C) buffer at 37°C [5,21]. The fluorescence intensity was monitored on a

Cytofluor 4000 Fluorescence/Bioluminescence Reader (excitation 485 nm, emission 590 nm) for the complete incubation period. ASP^+ uptake was calculated by subtracting the initial fluorescence at t=0 from the endpoint.

Transepithelial creatinine transport assay

Transepithelial transport of [^{14}C]-creatinine by matured ciPTEC monolayers cultured on polyester Transwell® inserts was measured. Mature cell monolayers were washed in modified Krebs-Henseleit buffer (Sigma-Aldrich Co., Zwijndrecht, the Netherlands) including 10 mM Hepes (pH was set to 7.4 using 1M HCL solution). Subsequently, all membranes tested were pre-incubated for 2 h at 37°C, 5% (v/v) CO_2 in Krebs-Henseleit-Hepes buffer (0.5 mL apically, 1.5 mL basolaterally). Transport was initiated by the basolateral addition of either [14C]-creatinine (0.75 µM, 2 µCi/ml) or [3 H]-inulin (0.45 µM, 20 µCi/ml) with or without digoxin (10 µM) or cimetidine (100 µM) as competitors for organic cation transport (Sigma-Aldrich Co., Zwijndrecht, the Netherlands). At the start of the measurement, a 20 µL reference sample was taken from the basolateral exposure compartment. After 15 and 30 min of incubation with gentle agitation at 37°C a 200 µL sample was removed from the apical chamber. The activity of [^{3}H] and [^{14}C] in the samples was determined by liquid scintillation counting (Beckman). Fluxes of [^{14}C]-creatinine or [^{3}H]-inulin were determined for each separate Transwell®. The flux of [^{3}H]-inulin was used as an internal leakage marker. The basolateral to apical flux (J) was calculated with the following formula:

$$J = \frac{dQ}{S * dt} \quad (1)$$

where dQ = amount transported [pmol]; dt = duration of transport [min]; S = surface area [cm^2].

Toxicity assay

An (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed to measure cellular toxicity of the compounds tested [22]. In short, cells were seeded onto culture dishes as described and cultured at 37°C for 7 days, under shaking or static conditions. The culture medium was then removed and the cells were exposed for 24 h to a UT mixture dissolved in culture medium at a concentration range from 1 to 1,000 fold the concentration reported in uremic patients. After incubation, the medium was changed to 5 mg/ml MTT in PBS and the plate was incubated for four h at 37°C. The intracellular accumulated precipitate was solubilized in 200 µL dimethylsulfoxide. The absorbance was read using a Dynatech MR 580 plate reader using a wavelength of 570 nm from which background was subtracted.

Detection of ROS formation

Cells were cultured on 12 well plates as described above. After washing the mature cell layers in modified Krebs-Henseleit buffer (Sigma-Aldrich, Zwijndrecht, the Netherlands) including 10 mM Hepes (pH 7.4), the ROS production was measured using the H2-DCFDA detection kit (Invitrogen, Bleiswijk, The Netherlands). This fluorescent probe detects several ROS variants including hydrogen peroxide, hydroxyl radicals and peroxynitrite. After entering the cell, H_2DCFDA is converted to the fluorescent 2',7'-dichlorofluorescein (DCF) due to oxidation by ROS. For this assay 1 mM H2-DCFDA was prepared in water-free DMSO (Sigma; D2650). This stock was further diluted (in Krebs-Henseleit Hepes buffer, 37°C) to reach a concentration of 10 µM. ciPTEC were pre-incubated with 500 µl H2-DCFDA (10 µM) for 30 min at 37°C at 5% CO2 (v/v). After incubation, the cells were washed once in warm Krebs-Henseleit Hepes. Subsequently, ciPTEC were treated with 1 mM H_2O_2 (positive control), 1x, or 10x UT-mix in Krebs-Henseleit Hepes buffer. Fluorescence detection was performed during 30 min, using a Victor X3 fluorescence plate reader at excitation 464 nm and emission 530 nm.

FACS analysis

Flow cytometry was used to study the expression of vimentin, a mesenchymal cell marker, and the presence of E-cadherin, a calcium-dependent cell-cell adhesion protein present in epithelia [23,24]. CiPTEC were seeded as described above in 12-well plates and allowed to adhere overnight at 33°C followed by maturation for 7 days at 37°C under shaking or static conditions. After this maturation period, cells were treated for 24 h with a cationic UT mixture, *viz.* spermidine 0.67 µM, spermine 0.09 µM, cadaverine 0.21 µM, putrescine 0.88 µM, acrolein 1.42 µM, guanidine 2.18 µM, and methylguanidine 7.66 µM [22,25-28]. CiPTEC were harvested using trypsin-EDTA and centrifuged at 600 x g during 5 min. The harvested cells were fixed and permeabilized using 4% (w/v) PFA and 0.1 % (v/v) saponin in HBSS on ice for 10 min. Supernatant was removed and the cell pellet was resuspended in 100 µL PBS containing 1 mL mouse-anti-human Vimentin-PE (Abcam, Cambridge, UK) followed by 30 min incubation at RT. For E-cadherin staining cell pellets were resuspended in rat anti-E-cadherin antibody (1:100 in HBSS) and incubated at room temperature for 30 min. Next, cells were centrifuged again and pellets were resuspended in goat-anti-rat Alexa 488 conjugate (1:200, Life Technologies Europe BV, Bleiswijk, The Netherlands) and incubated at room temperature for 30 min. After a final centrifuge step, cells were resuspended in HBSS buffer and measured using a flowcytometer (FACSCalibur BD, software BD CellQuest Pro version 6.0, Becton Dickinson, Franklin Lakes, NJ) gating on live cells (a total of 15,000 cells counted). Analysis was performed using Flow Jo software (TreeStar, Ashland, USA).

Data statistical analysis

Average values are depicted as mean ± SEM. Except for the qPCR data, where values are depicted as mean ± standard range. Statistical significance ($p<0.05$) was calculated using

Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA using an unpaired *t*-test or two-way ANOVA followed by Dunnett's post test where appropriate. ASP^+ uptake is shown as a percentage of the maximum. Nonlinear regression is calculated using the GraphPad Prism one binding-site competition. The presented curves are an average of three independent experiments. For the MTT assay, viability was expressed as a percentage of the values obtained from untreated control cells within the same experiment. MTT assays were performed in triplicate and repeated at least three times.

RESULTS

Dynamic cell culture conditions increase ciPTEC monolayer height and cilia length.

CiPTEC were cultured on collagen IV coated Transwell® polyester membranes, which permits the cells to form a polarized monolayer with clear apical and basolateral orientation [6,29]. After 7 days of culture, immunofluorescence microscopic analysis of the tight junction protein ZO-1 was performed (Figure 6.1A). This protein is considered a marker for the integrity and polarity of the epithelial cell layer. Both the ciPTEC cultured under static conditions, and those cultured on the 2D plate rocker formed a confluent monolayer. Furthermore, we examined the expression of primary cilia. Next to villi, primary cilia are important mechanosensory organelles that can sense tubular flow [30,31]. Confluent ciPTEC monolayers express primary cilia on their apical surface, as shown in Figure 6.1B and C. The α-acetylated tubulin network within the cell is visible, but the highest intensity was present in the cilium body. No significant differences were observed in the number of ciPTEC expressing cilia when cultured under static or dynamic conditions.

To evaluate the effect of dynamic culture conditions on cilia length, an antibody was used that specifically targets RPGRIP1L, a protein localized at the ciliary base. The fluorescent signal from this protein provided a clear starting point to measure cilia length under both culture conditions (Figure 6.1B, C). The monolayer height increased significantly, from 9.7 ± 5.2 μm to 15.5 ± 4.8 ($p<0.005$; Figure 6.1D). Dynamic cell culture on a 2D rocker did induce the formation of slightly longer cilia (6.9 ± 0.9 μm), compared to statically cultured cells (5.7 ± 0.4 μm, $p=0.01$; Figure 6.1E), though no differences were observed in TEER (Figure 6.1F).

Upregulation of membrane transporter mRNA expression in dynamically cultured ciPTEC

The mRNA expression levels of various proximal tubular transport proteins are depicted in Figure 6.2. For ciPTEC cultured on a plastic surface, higher expression of organic cation transporter 2 (OCT2) was observed when cultured dynamically on a 2D plate rocker (fold increase: 3.5 ± 2, $p=0.05$). Furthermore, ciPTEC grown on the 2D plate rocker expressed

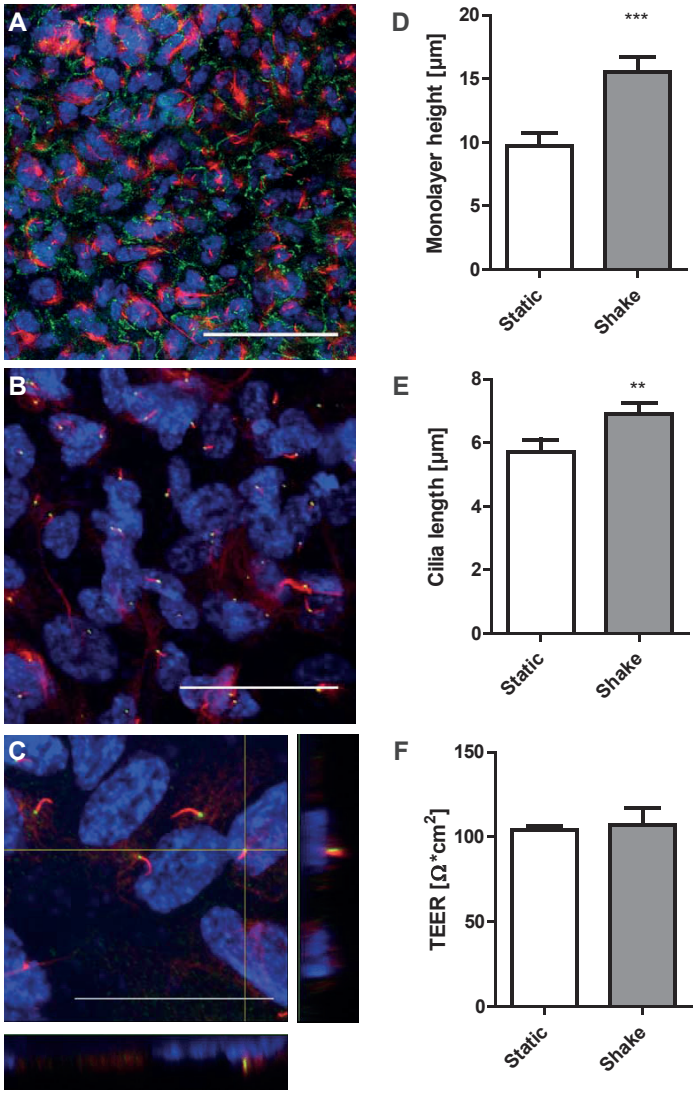

Figure 6.1: Representative images of ciPTEC cell morphology, expression of primary cilia, and ciPTEC epithelial cell characteristics under static or dynamic culture conditions.
(A) Immunofluorescence staining of the tight junction protein ZO-1 (green), α-acetylated tubulin (red) and nuclei (blue) after formation of a confluent monolayer magnification 60x. (B) Immunofluorescence staining of to visualize primary cilia, stained are α-acetylated tubulin (red) and the basal body protein RPGRIP1L (green), magnification 40x. (C) Zoom of cross-sectional views z-section images, stained were α-acetylated tubulin (red) and the basal body protein rp-grp (green). Note the apical localization of the primary cilia. (D) ciPTEC monolayer heights measured by confocal microscopy in z-sectioned images (N≥16 membranes). (E) Cilia length measured in z-stacked images using the RPGRIP1L basal body as starting point (N=8 confocal images). (F) Transepithelial electrical resistance measurements (N=12 membranes). Values are given as mean ± SEM *p<0.05, **p< 0.01.

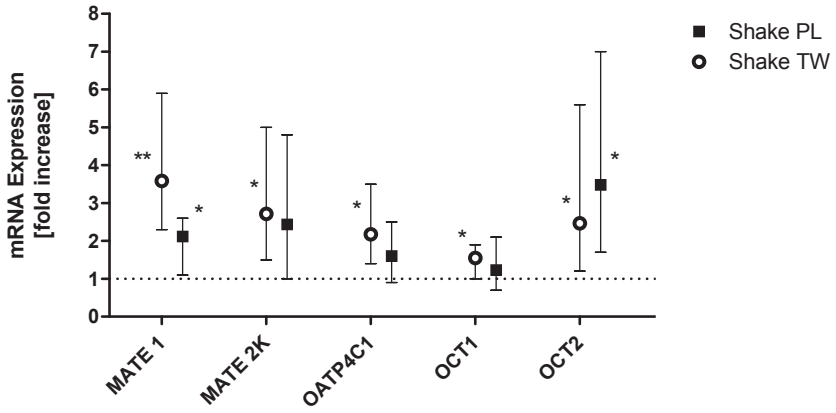

Figure 6.2: Cells cultured on a 2D plate rocker show increased mRNA expression levels of important cation influx (OATP4C1, OCT1, OCT2) and efflux (MATE1, MATE2K) transporters. Expression was measured in ciPTEC cultured on a 12 wells culture plate (■, PL) or in ciPTEC monolayers cultured on Transwell® membranes (**o**, TW). Average results are shown of three separate measurements in duplicate, values are given as mean ± standard range *p<0.05, **p< 0.01.

increased mRNA levels of multidrug and toxin extrusion protein 1 (MATE1) (fold increase: 2.1 ± 0.6, p=0.02). Remarkably, when the same experiment was performed with ciPTEC cultured on polyester Transwell® membranes, a significant increase was also observed for MATE1, MATE2K, solute carrier organic anion transporter family member 4A1 (SLCO4C1), and OCT1 (Figure 6.2).

Increased transmembrane organic cation transport by ciPTEC cultured on a 2D plate rocker

Next, we evaluated if the observed increased transporter expression translated to enhanced organic cation uptake. Using a fluorescence-based method, the uptake of the substrate ASP^+ was evaluated [21,32]. Figure 6.3A demonstrates the concentration dependent ASP^+ uptake, which increased up to 151 ± 28% (p<0.05) in matured ciPTEC cultured under shaking conditions compared to statically cultured ciPTEC. Inhibition of ASP^+ uptake with the potent inhibitor tetra-pentylammonium (TPA) resulted in a maximum inhibition of 75%.

Transepithelial transport of [^{14}C]-creatinine was measured in presence and absence of cimetidine and/or digoxin, known inhibitors of organic cation transporters (OCTs, MATEs), and SLCO4C1, respectively [7,9,33-35]. Figure 6.3B presents the transmembrane flux of [^{14}C]-creatinine (0.75 μM) and [^{3}H]-inulin (0.45 μM) by ciPTEC cell monolayers. The paracellular leakage of [^{3}H]-inulin across the monolayer was used as an indicator of monolayer tightness since passive diffusion is the primary mode of transport for this compound [36]. The creatinine flux through the ciPTEC monolayers cultured under shaking conditions (2.9 ± 0.4 pmol $min^{-1}cm^{-2}$) was 1.4x higher than the flux (2.1 ± 0.4 pmol $min^{-1}cm^{-2}$) measured under

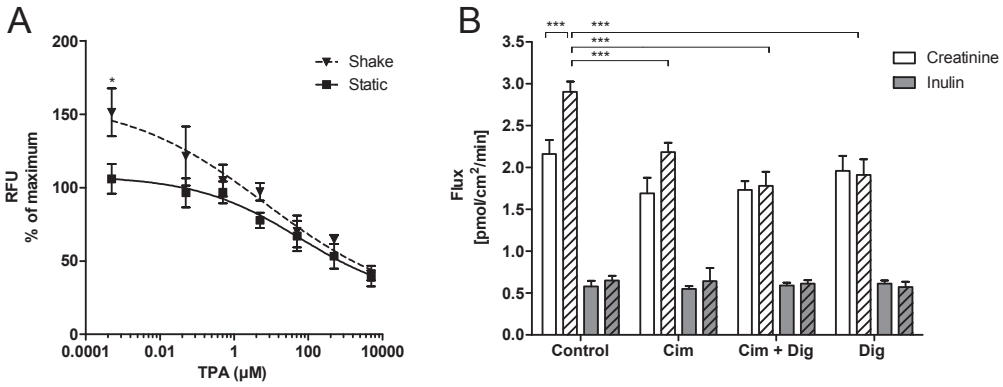

Figure 6.3: CiPTEC cultured on a 2D plate rocker show increased organic cation transmembrane transport.

(a) Concentration-inhibition curves of ASP^+ uptake by tetra-pentylammonium (TPA), in ciPTEC cultured on a shaking (▼) platform or under static (■) conditions. Values are given as mean ± SEM described as percentage of ASP^+ uptake. Presented curves are an average of three independent experiments. (b) Basolateral to apical fluxes of [14C]-creatinine (0.75 μM, white) and [3 H]-inulin (0.45 μM, grey) across ciPTEC monolayers cultured on collagen IV coated Polyester Transwell® membranes, using a 2D plate rocker (dashed) or under static conditions (clear). Measurements were performed in the presence or absence of the inhibitors cimetidine (Cim, 100 μM) or digoxin (Dig, 10 μM). Data are presented as mean ± SEM, N=3. Statistical significance: between static and shaking conditions, or between the cells cultured on a shaking platform *** $p<0.005$.

static conditions ($p<0.001$). The creatinine flux was significantly decreased by addition of cimetidine (static: 17%, $p<0.005$, shaken: 25% $p<0.001$), indicating active transport of creatinine. For the ciPTEC monolayers cultured under dynamic conditions, addition of digoxin, a selective inhibitor of the SLCO4C1 transporter, further stimulated inhibition of creatinine transport (41%, $p<0.005$). Maximal inhibition (44%, $p<0.005$) was reached when the combination of cimetidine and digoxin was applied. The inulin leakage was similar between the two conditions, indicating comparable cellular monolayer integrity.

Cationic UTs show decreased viability in dynamically cultured cells

Since OCT expression and functionality increased in ciPTEC cultured on a 2D plate rocker, we investigated whether a selection of cationic UTs affected the viability of these cells under shaking or static conditions. Previously, this selection of UTs showed to inhibit OCT mediated transport in ciPTEC, likely due to competitive inhibition [7]. Here, we exposed ciPTEC, either statically or on a plate rocker, to a mixture of toxins (UTmix) comparable to 1000, 100, 10, 1, 0.1 and 0.01 times the uremic plasma concentrations reported in literature; *viz.* spermidine 0.67 μM, spermine 0.09 μM, cadaverine 0.21 μM, putrescine 0.88 μM, acrolein 1.42 μM, guanidine 2.18 μM, and methylguanidine 7.66 μM [25-28]. After 24 h of incubation with the

UT mixture, cells cultured on the rocking platform proved more sensitive to its toxic effects (Figure 6.4). The IC_{50} value calculated from the inhibition curve was significantly higher for the statically cultured cells (IC_{50}: 27.9 ± 2.7 times the UTmix) compared to shaking conditions (IC_{50}: 16.8 ± 2.3) (p<0.01).

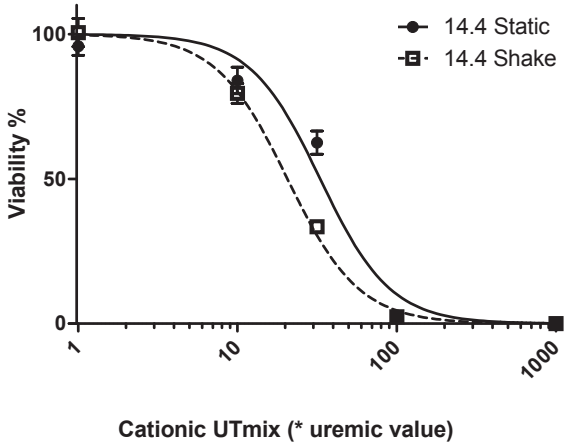

Figure 6.4: ciPTEC viability after 24h of exposure to a UT mixture under shaking (□) or static (●) conditions.
CiPTEC were exposed to mixtures of selected cationic UTs. Toxin concentrations were taken at 1000, 100, 10, 1, 0.1 and 0.01 fold the uremic plasma concentrations reported in literature [25-28]. Viability was determined by the MTT assay, performed in triplicate. Results are shown as mean values ± SEM.

No increase in ROS production.

Since it is known that dynamic culture conditions can promote ROS formation [37], CM-H_2DCFDA measurement was performed. Basal ROS production did not differ between the two culture conditions (Figure 6.5). After addition of the ROS inducing agent H_2O_2, a time dependent ROS production was measured. Although the overall ROS production in the dynamically cultured cells appeared higher than under static conditions, the difference was not statistically significant (p=0.06). ROS production after exposure to the 1x or 10x mixtures of UTs for 30 min did not differ from the unexposed cells. Furthermore, dynamic cell culture in the presence or absence of UTs had no significant effect on vimentin or E-cadherin expression levels (data not shown).

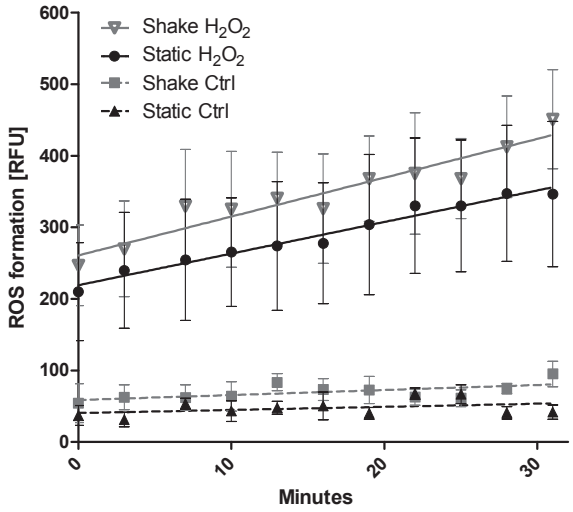

Figure 6.5: ROS formation by ciPTEC monolayers cultured under dynamic or static conditions. ROS production was measured using the H2-DCFDA detection kit in: untreated (dashed line) or H2O2 treated (solid line) cells cultured statically (black) or on a 2D plate rocker (grey) in the presence of the DCFDA probe (8 mg/ml). DCFDA fluorescence is depicted as relative fluorescence units in triplicate of three experiments.

DISCUSSION

In an effort to elucidate the effects of simulated shear stress on ciPTEC monolayer quality, as well as the transepithelial creatinine transport capacity of the proximal tubular epithelial cell monolayer, we simulated shear stress by culturing ciPTEC on a 2D plate shaker. By using this method to induce fluid flow on the epithelial surface, mRNA expression of various transport proteins, as well as the transepithelial creatinine membrane transport increased. Furthermore we observed enhanced sensitivity to cationic UTs in cells cultured on the 2D plate shaker.

Cell models are commonly used to simulate and study processes taking place in organ systems in the human body. Culture methods are constantly being refined to enhance cellular properties to better approximate the physiological situation. During the last 30 years, various *in-vitro* culture systems have been developed that would provide suitable supports for the formation of epithelial monolayers. More advanced culture systems aim to include an element of flow to produce shear stress comparable to the physiological situation; thereby stimulating further differentiation of cultured cells. In mouse proximal tubule, a shear force of 1 Dyn/cm^2 has been reported [14]. However, a more recent study that modeled shear in a laminar flow chamber based on tubular fluid velocity resulting from single nephron glomerular filtration rate, reported a much lower mechanical stress of 0.17 Dyn/cm^2 [15]. In the present study, the 2D-plate rocker was able to simulate a characteristic shear stress on cultured ciPTEC

monolayers grown on Transwell® membranes up to 0.26 Dyn/cm^2, which is in the range of previously reported values. The use of such a shaking platform provides an easy to perform method to simulate flow. Multiple conditions can be measured at the same time without the use of an intricate setting involving a myriad of pumps, tubings and connectors. Furthermore, similar culture dishes and media volumes can be used for the static control situation as for the flow stimulated cells, making the conditions highly comparable. However, an important limitation that should be taken into account is the lack of unidirectional flow. Various studies have indicated that bending of the primary cilium relays signals from the extracellular compartment into the cell. For the renal tubule, this regulatory process has been shown to depend on Ca^{2+} signaling [38], and the modification of signaling pathways such as WNT [39-41], JAK-STAT [42], or mTOR [43]. However, on a 2D plate rocking system, the change in direction could also affect cellular signaling processes. It would therefore be very interesting to compare the activation of these different signaling pathways under unidirectional and bi-directional flow conditions. Furthermore, the use of ciPTEC monolayers grown on Transwell® inserts on a 2D plate rocking system might be limited to short term exposure experiments, as this model does not provide clearance of waste products produced by the cells, which other flow devices might offer.

In the present study, dynamic culture conditions increased ciPTEC monolayer height and induced lengthening of the primary cilia. Monolayer height is used as a marker for the degree of polarization within an epithelial layer, since a well-differentiated proximal epithelial monolayer consists of tall columnar cells with a clear apical and basolateral orientation. We did not observe any change in the TEER for monolayers cultured on the 2D plate rocker. The resistances measured here are comparable to the values reported for confluent PTEC monolayers in literature, indicating intact monolayer integrity [44]. The observed lengthening of the primary cilia on the cell monolayers cultured under flow conditions might indicate activation of flow-induced differentiation. That cilia are essential for proper tubular epithelial morphology was shown in studies of polycystic kidney disease (PKD). In PKD, ciliary shortening and protein defects are key contributors to loss of tubular phenotype and renal cyst development [45,46]. Furthermore, studies in mouse and human renal proximal epithelia reported primary cilia lengthening during recovery from either ischemia reperfusion injury, or acute tubular necrosis [47,48]. A cilium-based signaling pathway was presented, that mediates epithelial repair and differentiation necessary to restore a functional epithelial layer. This epithelial redifferentiation is proposed to be activated by the return of urine flow, which induces increased Ca^{2+} signaling, activation of non-canonical Wnt and the downregulation of both mTOR and STAT6 pathways [49,50]. This flow induced transition from a proliferative to a more differentiated cellular state, might also be initiated by inducing fluid flow in ciPTEC monolayers. However, further investigation into the activation of these signaling pathways in ciPTEC is necessary to evaluate the mechanisms behind flow induced monolayer differentiation.

Flow induced epithelial redifferentiation may promote an increase in solute transporter expression levels. Here we observed increased mRNA expression levels for various organic cation transporting proteins, after 7 days of culture under flow conditions. The effect was more pronounced in ciPTEC monolayers cultured on semi-permeable Transwell® membranes, which already promote epithelial differentiation into a polarized monolayer. Such a stimulating effect of fluid flow on cellular functionality and solute transport was seen before in a microfluidic device developed by Jang et al [18]. In the present study bidirectional dynamic flow induced a similar enhancement of cation transporter activity. This indicates that culturing ciPTEC on a 2D rocker may stimulate organic cation uptake.

Proximal tubular solute uptake is an important first step in the active removal of organic solutes from the circulation. However, subsequent apical excretion of these compounds is crucial for efficient drug or solute clearance. By measuring the transepithelial transport of [^{14}C]-labeled creatinine, we examined if the clearance processes in this ciPTEC model are affected by fluid flow. While in clinical practice creatinine is used as a marker for glomerular filtration rate, proximal tubular secretion can account for 10 to 20% of urinary creatinine excretion [51]. The significant increase in transepithelial transport of this endogenous compound in dynamically cultured ciPTEC monolayers is in line with the observed increased mRNA transporter expression levels. The higher efficiency by which both cimetidine and digoxin inhibited the creatinine transport in cells cultured under dynamic conditions, further support a more active or prominent role of the transport proteins such as OCTs [52] and OATP4C1 [35].

Various studies have reported shear stress or fluid flow induced oxidative stress in cultured vascular cells [53,54]. The measurement of the oxidation of H_2DCFDA by hydroxyl radicals or hydrogen peroxide molecules in the present study, indicated that culturing ciPTEC on a 2D plate rocker does not significantly stimulate additional production of reactive oxygen species. Although UTs are known to promote the formation of reactive oxygen species [37], the addition of a cationic toxin mixture did not induce formation of hydroxyl radicals in ciPTEC. Based on these findings, the observed viability decrease is therefore unlikely to be mediated by oxidative stress. We should, however, take into account that the viability was measured by using the MTT viability assay, which is based on measuring mitochondrial function, while H_2DCFDA detects all cellular ROS and does not target mitochondrial functioning specifically. Mitochondrial ROS detection, for example by using dihydroethidium (MitoSOX) probes, might provide more insight into the specific mitochondrial ROS production. The increased sensitivity of ciPTEC to UTs could be related to an increase in epithelial character of the monolayer. Tubular epithelial cells are prone to a de-differentiation process known as Epithelial to Mesenchymal Transition (EMT). In EMT, tubular epithelial cells develop a more fibroblast-like character in response to cellular stress [55,56]. The fibroblast like character can render the cell more resistant external assaults, and stimulates migration as well as proliferation [57]. Although fluid flow is thought to stimulate ciPTEC to retain their epithelial

characteristics during exposure to UTs, no significant effects on the respective mesenchymal and epithelial markers vimentin or E-cadherin were detected after 24 h of exposure, indicating no induction of EMT by this set of cationic UTs.

The human ciPTEC model is a valuable tool to acquire mechanistic insights in renal clearance of uremic retention solutes. In previous studies, the renal epithelial characteristics and cationic toxin handling of this cell model were characterized extensively [5,6,8,9] . Furthermore, science has been exploring the possibilities to utilize the functional properties of renal cells, in terms of their active role in the clearance of uremic retention solutes, in an add-on device to renal hemodialysis therapy, a bioartificial kidney, or so called Renal Assist Device (RAD). Such a RAD would consist of a cassette that contains multiple semi-permeable hollow fibers, or membranes, on which tight cellular PTEC monolayers are grown. By attaching such a device in series with an existing hemodialysis unit, the dialyzed blood flows over these "living-membranes", thereby enabling further active clearance of uremic waste products. Our results indicate that the application of fluid flow can improve the RNA expression of various cation transporters, cellular sensitivity and increases transepithelial creatinine transport. Though we observed increased ciPTEC toxicity after 24h incubation with UTs in 12 wells plates under dynamic culture conditions, it would not have to mean similar toxic effects would take place in a bioartificial device. The 12 wells plate set up cannot not take into account the presence of continuous waste removal, which would be an important feature of a RAD. Furthermore, we also observed mRNA up-regulation of various efflux proteins, which could lead to a faster clearance of UTs in a continuously perfused system. However, as mRNA levels do not always directly correspond to protein expression, it would be sensible to investigate the absolute expression levels as well as functionality of these transporters. For accurate measurements of the actual intracellular accumulation of these retention solutes, specialized LC-MS/MS based techniques may provide a valuable tool in the detection of these compounds.

In conclusion, culturing ciPTEC under fluidic conditions on a 2D plate rocker stimulated the endogenous transporter functionality of the cell monolayer. The increased transporter expression and transport observed under dynamic culture conditions, indicates that the use of a relatively simple system to model fluid flow can have a positive effect on cell functionality. Enhancing the endogenous organic cation transport of ciPTECs could make this cell line even more interesting to investigate renal clearance processes in pharmacological and physiological studies. Furthermore, transepithelial solute transport is an important feature in the development of innovative bioartificial renal replacement therapies. The positive effect of flow on proximal tubular transepithelial transport could provide an extra rationale for the development of a flow based RAD.

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7

Discussion

INTRODUCTION

The renal proximal tubule is a versatile part of the nephron, which plays a crucial role in the active secretion and reuptake of numerous exogenous as well as endogenous compounds. These processes are mediated by a wide variety of transport proteins, present in the basolateral and apical membranes of proximal tubule epithelial cells (PTEC). During chronic kidney disease (CKD), renal transport processes become disturbed, resulting in the systemic accumulation of waste products. The increase in knowledge on the uremic retention solutes that accumulate in CKD patients broadens our understanding of renal disease etiology and disease progression. These insights stimulate investigations towards improving the removal of specific uremic retention solutes by renal replacement therapy. To this end, we studied the handling and transport of cationic uremic solutes by ciPTEC. In this chapter, the implications of these findings are discussed and placed in the perspective of future research towards kidney functioning and the possible development of a bioartificial kidney or renal assist device (RAD) for the treatment of CKD.

OPTIMIZING RENAL CELL MODELS TO STUDY CLEARANCE PROCESSES IN THE NATIVE KIDNEY

Renal cell models are widely employed to study renal clearance and physiology [1-3]. While *in-vivo* studies are often indispensable for providing insight into the clinical relevance of observations made with *in-vitro* techniques, they are not as easily controlled. Interspecies variability and extrarenal influences, such as body temperature, hormone levels, nervous regulation, hemodynamics, and glomerular filtration rate (GFR), can complicate the interpretation of the data. By making use of *in-vitro* models, many of these factors are taken out of the equation and enable the study of very specific processes in a high throughput fashion. Depending on the level of preservation of renal functionality, cell models can help in unraveling renal physiology and clearance processes, and mechanisms leading to renal toxicity and failure. Furthermore, these cell models might prove useful in novel bioartificial treatment applications.

To study the function of renal cells *in-vitro*, cell models should reflect renal physiology. It is therefore essential to characterize the nephron-specific properties, such as polarized transport, reuptake of solutes, and the formation of tight monolayers, in both established and novel cell lines. While established human renal epithelial cell lines, such as HK-2 or HEK293, are frequently employed in the study of renal processes [4,5], their low (endogenous) transporter expression levels make these models less suitable for tissue engineering purposes [6,7]. More recently, a conditionally immortalized human PTEC cell line (ciPTEC) was developed from human urine samples by Wilmer *et al.* [8]. This ciPTEC model expresses several functional in-

and efflux transporters, and can perform active albumin and sodium-dependent phosphate uptake. Due to its functional proximal-tubular characteristics and relative ease of obtaining large cell numbers, this cell model was used as a tool in studying renal pathophysiology [9,10], metabolism [11] and transport pathways (Chapters 3-6). As ciPTEC originated from exfoliated cells isolated from a human urine sample, it may be argued that they would not make the ideal source for the establishment of a PTEC model. However, our functional and morphological comparison between the established ciPTEC line and two newly immortalized cell lines obtained from kidney tissue, revealed comparable functional characteristics (Chapter 2). The differences between these cell lines were found mainly in cell size and extracellular matrix (ECM) gene expression levels. The observed lower collagen I and -IV α1 expression in the cell lines originating from urine as compared to tissue-derived ciPTEC, may have been the result of the exfoliation process preceding their excretion [12,13]. This finding stresses the importance of primary cell source and isolation method on cell line characteristics. The somatic cell from which immortalized cell lines were developed, could have influenced the *in-vitro* characteristics of differentiated cells through epigenetic processes [14]. Epigenetic effects have been observed in pluripotent stem cell research, where the transient epigenetic memory of the somatic cells of origin influenced differential gene expression and altered differentiation capacity [15]. As described in this thesis, methods to screen potential cell lines for tissue specific characteristics involves the use of a large variety of testing methods and can therefore be time consuming. In Chapter 2, the ciPTEC cell lines were characterized using a combination of gene and protein expression arrays, immunocytochemistry, and functional tests. These methods provided important information about the type and quality of the cell lines developed. However, the (limited) availability of specific antibodies to detect the expression levels of cell-type specific proteins hampers true identification. Proteome profiling, a recent development in cell type identification, might in future simplify the optimization of the cell system [16]. This technique is not dependent on antibody availability, specificity or quality [17], and could potentially be used to examine both the type, variant and quantity of transporters expressed in the models [18].

In vitro culture conditions

Next to the selection of a well-characterized and functional cell type, cell culture conditions are key factors in studying renal clearance processes. In current practice, cells are mostly cultured statically and on plastic surfaces. Though these conditions are hardly organotypic, they often provide satisfactory results. However, for the development of a bioartificial RAD, studies on directional transepithelial clearance processes may be very important. While Chapters 3 and 4 have shown that uptake of organic cations can be modeled in cell suspensions or on cell monolayers grown on plastic surfaces, the examination of transepithelial (uremic) solute transport required the use of a polarized cell system. By culturing monolayers of epithelial cells on semi-permeable membranes, such a polarized system can be obtained (figure 7.1).

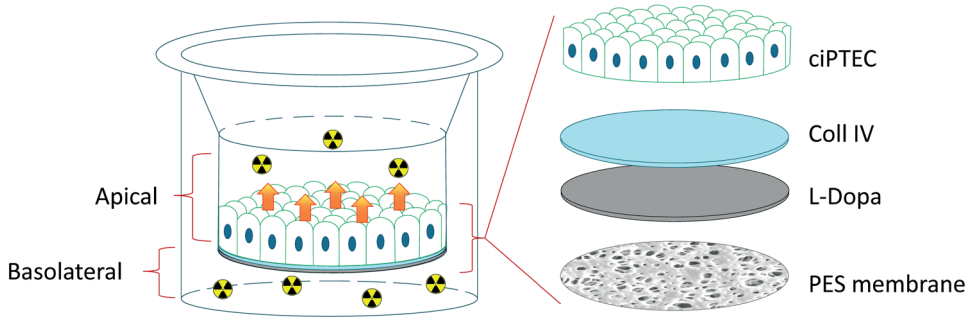

Figure 7.1 Schematic overview of the Transwell® set up.
Set up as used for the examination of basolateral to apical creatinine transepithelial transport and inulin leakage across ciPTEC monolayers. Radiolabeled substrates are indicated with the radioactive trefoil symbol, with the arrows indicating the direction of transport.

The semi-permeable culture substratum, consisting of the ECM and the physical tissue culture support, has been identified as an important factor in influencing cell shape, size, attachment and degree of differentiation [19-24]. By culturing epithelial cell monolayers on specialized permeable surfaces, the functional differentiation of epithelial cells could be increased [25,26]. Commercially available polyester or polycarbonate semi-permeable surfaces are often used in the study of polarized cell systems [27]. However, as these materials are designed to promote ECM deposition and cell attachment, they are less suitable for application in a clinical device. Therefore, hemocompatible materials that are used in dialysis and/or blood purification therapy would provide promising alternatives. Several studies evaluated polyethersulfone (PES), polysulfone (PSF), polyacrylonitrile (PAN) and cellulose acetate membranes for their ability to support monolayer growth of renal epithelial cells [28-30]. In Chapter 5 we showed that ciPTEC are able to form polarized and functionally active cell monolayers on PES-based semi-permeable dialysis membranes by making use of an optimized coating, consisting of a thin adherent poly-dopamine (PDA) film to which the biomolecule collagen IV was covalently bound. This double-coating strategy promoted cell attachment to the surface of the dialysis membrane [31]. The principle of membrane bioactivation for the development of renal epithelial monolayers had been described previously [32-34]. However, careful evaluation of the membrane properties before and after coating was necessary, as addition of these extra layers should not obstruct the pores of membrane. In the case of the optimized coating as described in Chapter 5, the PES membranes tested retained permeance, and facilitated functional transepithelial transport of creatinine, with minimal passive inulin diffusion. In fact, we were able to show functionality of the transepithelial creatinine secretory pathway in confluent ciPTEC monolayers grown on these optimized PDA and collagen IV coated PES membranes (figure 7.1).

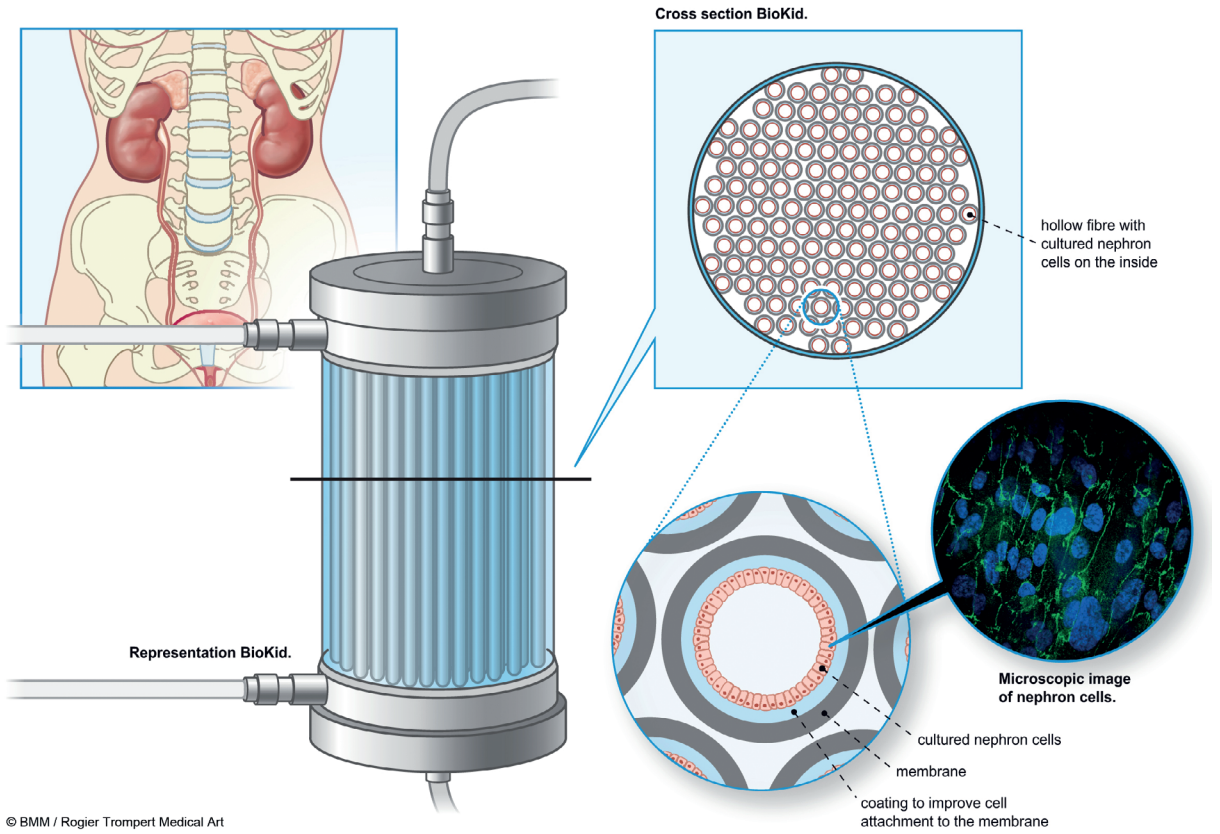

Figure 7.2 Proposed set-up for a bioartificial kidney or RAD.
Bioartificial kidney or RAD consisting of a cassette containing multiple semi-permeable hollow fibers. In this proposed set-up the tight cellular PTEC monolayers are grown on the inside of coated hollow fibers. Reprinted with permission © BMM / Rogier Trompert Medical Art.

The current set up for such a RAD would consist of a cassette containing multiple semi-permeable PES-based hollow fibers, or membranes, on which tight cellular PTEC monolayers are grown (Figure 7.2). By attaching such a device in series with an existing hemodialysis unit, the dialyzed blood flows over these "living-membranes", thereby enabling active secretion of uremic waste products. Altering the culture conditions to a more dynamic and organotypic set up is known to stimulate cell differentiation [35]. As shown in Chapter 6, the application of fluid flow improves the expression of various cation transporters, cellular sensitivity and increases transepithelial creatinine transport. These findings suggest that the introduction ciPTEC to apical shear-stress can stimulate further differentiation of this cell type. The rocking system used is a simplified model for simulating epithelial fluid flow. Though flow can be simulated easily in such a rocking model, it cannot provide unidirectional or continuous perfusion. Various sophisticated microfluidic culture systems are currently being developed that permit continuous perfusion of culture media over cellular monolayers [36-38]. The use of these systems for the culture of epithelial cells could further stimulate differentiation and maintenance of an *in-vivo* like state over prolonged periods [39]. Though we obtained promising results using our simplified 2D rocking model, additional research is required to examine if living ciPTEC membranes are able to withstand unidirectional fluid flow whilst keeping the monolayer intact to prevent leakage. Furthermore, would be important to determine the functionality and clearance capacity of ciPTEC inside a bioreactor or RAD prototype, both during short term and long term culture.

UREMIC SOLUTE HANDLING BY CIPTEC

In the healthy kidney, the clearance of many of the protein bound solutes occurs with high efficiency through active secretion [40]. However, over 150 solutes have been identified to accumulate systemically in patients suffering from ESRD [41,42]. Increasing knowledge on the importance of uremic waste product accumulation in the progression of CKD has led to an interest in the renal handling of uremic solutes. As described in Chapter 1, basolateral uptake and apical efflux are crucial for proximal tubular transepithelial secretion processes. Harnessing these functions to allow removal of uremic solutes from the circulation in a RAD requires insight in their cellular handling.

Uptake of uremic solutes

CiPTEC express a myriad of proximal tubular characteristics, as illustrated in Chapter 2. Functionality was shown for a variety of endogenously expressed organic solute transporters, such as OCT2, P-gP, MRP4 and BCRP. Their presence makes it possible to examine physiological transport processes. However, a drawback of the ciPTEC model used, is the absence of the organic anion transporters OAT1 and OAT3. In cultured proximal tubule epithelial

cells, rapid loss of OAT expression is commonly observed [43]. Their endogenous expression has not been observed in a PTEC line so far. Therefore, studies regarding the functionality of these transporters rely mostly on overexpression systems [44]. For the development of a bioartificial RAD, their absence is also considered a substantial limitation, because of their recognition as important transport proteins in the removal of anionic solutes [45,46]. However, such changes in phenotypic expression may be turned into an advantage when a specific detail of a complex process is studied. For instance, if a uremic solute is taken up by several carriers *in-vivo*, the absence of one of them in a cell line allows studying the other(s) more selectively. Though the eventual translation of results to the physiological situation will become more challenging.

CiPTEC express a functional set of endogenous organic cation transporters. Chapter 3 describes the validation of a cell suspension-based method, by which inhibition of the OCTs by uremic waste products was detected. The use of such a method enables testing of a range of solutes in a high throughput way, while keeping cellular integrity intact. For investigations towards transporter functionality this method has proven very effective [47-49]. As described in Chapter 3, uremic toxins (UTs) belonging to the group of guanidino compounds, polyamines, and the breakdown product acrolein, interfere with OCT-mediated uptake in ciPTEC (Chapter 3). Acrolein, a polyamine oxidation product [50-52], was the only solute for which a complete inhibition curve could be obtained. Acrolein inhibited the influx process in a competitive way. These observations suggest that endogenous OCTs expressed by proximal tubular cells can functionally mediate the influx of cationic UTs.

Similar findings have been reported for the proximal tubular transport of anionic uremic solutes. In 2012, Uwai Y *et al.* reported interaction of kynurenic acid with human organic anion transporters hOAT1 and hOAT3 [46]. More recently, p-cresyl sulfate was reported to be a good substrate for hOAT1 and hOAT3 [53]. In ciPTEC, because of the OATs' absence, the uptake of these compounds is likely fully dependent on diffusion or on the endogenously expressed SLCO4C1 transporter [54]. In 2009, Toyohara *et al.* illustrated the importance of the renal SLCO4C1 transporter in the elimination of UTs thereby attenuating hypertension and renal inflammation [55]. In rats, SLCO4C1 overexpression decreased plasma levels of the cationic UTs guanidino succinate, asymmetric dimethylarginine, and trans-aconitate.

Transmembrane transport

While uptake of organic retention solutes through transporters, such as OCT and SLC04C1 is the first step in the removal of organic solutes from the circulation, their secretion into the tubular lumen is dependent on the collaboration between the basolateral influx transporters and proper functioning of apical efflux transporters. Various studies reported active transepithelial transport of endogenous compounds or xenobiotics, such as creatinine, digoxin, metformin and cimetidine [56-59]. However, limited data are available that describe *in-vitro* transepithelial uremic solute transport by human PTEC monolayers. The most common

approach to study this type of transport, is to use radiolabeled drugs such as verapamil or metformin, as model compounds [58,60]. For example, Brown *et al.* reported transepithelial fluxes of the prototypic model substrates PAH and creatinine by primary PTEC [56].

The observed active transepithelial creatinine transport described in Chapter 5 indicates the presence of functional endogenous organic solute secretion processes in ciPTEC. Furthermore, with inulin as a leakage marker, cell monolayer tightness was confirmed, since active transcellular transport has not been reported for this compound [61]. However, further insight in the removal of specific cationic (and anionic) uremic waste products is necessary to evaluate if ciPTEC grown on PES-50 surfaces could potentially be used in the clinic. Application of sophisticated bioanalysis techniques, such as LC-MS/MS, might provide opportunities for the transepithelial transport measurements of a large number of uremic solutes by ciPTEC monolayers [62-64].

Investigating monolayer functionality under uremic conditions

As shown in Chapter 4, exposure of ciPTEC to a uremic milieu can initiate production of IL-6, IL-8 and ET-1, indicating the commencing of an inflammatory response. Inflammation is often observed in patients suffering from ESRD, and uremia has been identified as an important causative factor [65-67]. In ciPTEC, ET-1 was found to differentially regulate organic cation uptake through the ET-1-ET-B receptor axis. Both inducible nitric oxide (iNOS); a known mediator of local inflammatory responses during endotoxemia [68,69], and protein kinase C (PKC), were identified as important signal transducers. These mediators attenuated the ET-1 induced inhibition of organic cation uptake after 30 min, but had no effect after 24 h. These observations were reminiscent of both the biphasic and NO-independent pathways described for the efflux transporter P-gp [70,71]. For P-gp, a second pathway, next to NO mediated regulation, was identified involving activation of Toll-Like Receptor 4 and translocation of NF-κB [70,71]. Further research would be warranted to investigate whether (cationic) UTs could also regulate proximal tubular transport processes through this pathway. Recent studies have already reported down-regulation of SLCO4C1 transporter expression after exposure to the anionic uremic solutes indoxyl sulfate, p-cresyl-sulfate and p-cresyl glucuronide [72]. On the other hand, the upregulation of efflux transporters, such as BCRP, was reported after 48 h of exposure to these compounds [72,73]. Such regulatory effects are thought to be part of a renal protective mechanism, which has also been observed upon exposure of renal cells to nephrotoxicants [69,74,75]. Taken together, our findings indicate that uremic solutes should be considered as more than just substrates. They are able to influence ciPTEC functionality not only by (competitive) inhibition of transport, but also through secondary regulatory pathways.

These observations, together with the observed toxicity of UT mixtures (Chapter 3) rationalize the need for further research towards ciPTEC monolayer stability under uremic conditions. A known phenomenon that should be taken into account in renal epithelial cells

exposed to uremic stress and inflammatory mediators, is epithelial-to-mesenchymal transition (EMT). Rastaldi *et al.* [76] detected the occurrence of proximal tubular EMT in human renal biopsies of different renal diseases. *In-vitro* studies have indicated that this process also occurs during uremic toxin exposure in experimental set ups, and thereby reduces the epithelial and transport characteristics of the epithelial cell lines tested [72,77,78]. No EMT was observed in the research described in this thesis, but cells were exposed to a selection of cationic uremic retention solutes in an experimental setting. Hence, it would be prudent to examine the effect of ciPTEC exposure to human uremic plasma, as uremic plasma comprises a much more complex mixture of organic and anorganic solutes. Furthermore, various studies are being set up in order to overcome EMT, and stimulate the maintenance of an epithelial phenotype of renal cells in culture. One strategy might be to utilize the mechanism that was discussed by Al-Lahham *et al.* [79]. In that study, a positive effect of culture media hypertonicity was demonstrated. Their findings suggested that stimulation of the tonicity-responsive enhancer binding protein (TonEBP) could be used to maintain the epithelial phenotype of renal epithelial cells in culture.

Another sophisticated strategy for the improvement of monolayer stability, could involve the incorporation of bio-active molecules into the membrane scaffold [80]. In such bioactive membranes supramolecular polymers are used, which assemble into nano-fibers. Meshes are then formed by electro-spinning [81]. The bioactivity is incorporated in these nano-fibers by the addition of different ECM-peptides, such as collagen I, IV, fibronectin, and laminin, for stable binding [82]. In a study by Dankers *et al.* [83], tight and functional primary human renal tubular epithelial cell monolayers could be maintained on such bioactive meshes during a 19 days culture period. Incorporation of specific bioactive compounds into membranes could also be used to stimulate antifouling and cell differentiation. However, this type of membrane would also require optimization for the culture of renal proximal tubular cells such as ciPTEC.

TOWARDS CLINICAL APPLICATION

The proof of concept regarding ciPTEC monolayer function on coated flat PES membranes is the preliminary step towards the development of intricate prototype bioartificial devices made with bundles of multiple hollow fibers (figure 7.2). However, a long road of research lies ahead of us before such a concept can be applied in a pre-clinical or clinical setting. Integration of both synthetic and biological components into a single device brings along many challenges regarding the design, engineering, its functionality and durability, and can be associated with safety concerns.

The development of cell-based treatments in the field of tissue engineering is still gaining in popularity [84,85]. Somatic cell therapy medicinal products, gene therapy medicinal products, and tissue-engineered products are being developed for a wide variety of defects and

illnesses. These "advanced therapy medicinal products" (ATMPs) will have to be approved by the various international regulatory boards, prior to marketing [86]. ATMPs need to follow strict regulation with regard to the origin of cells, the level of cell manipulation, extent of replication competence of viruses, long time functionality, ability to proliferate or differentiate, to initiate an immune response, the risk of oncogenicity, *in-vivo* potency, and mode of administration or use. It might seem premature to consider these aspects in such an early stage, as the aim of this work was to examine the transport and uremic solute handling by ciPTEC. However, during the research phase it is still relatively easy to apply and test changes to the system. If we recognize what is possible in a simple system, we can start to translate these findings into a more complex arrangement and, eventually, utilize this knowledge for the development of an artificial device.

SAFETY ASPECTS OF GENETICALLY MODIFIED CELLS

A tissue-engineered bioartificial kidney or RAD, designed as a combined ATMP, would consist of a medical device, which follows the same basic principles as a dialysis unit, but is combined with viable renal epithelial cells that are substantially modified. In the case of ciPTEC, this manipulation involved the immortalization procedure performed using retroviral transduction of primary cells with two oncogenes [8]. Retroviral infection using both the essential catalytic subunit of human telomerase (hTERT) and the temperature-sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) is a recognized effective method for the development of conditionally immortalized cell lines [87-89] The SV40T gene allows cells to proliferate at temperature of 33°C, whereas at 37°C inactivation of the large T antigen permits cellular differentiation [90]. Addition of the hTERT vector increases the cellular telomerase activity, resulting in maintenance of telomere length, thereby avoiding the occurrence of replicative senescence [91,92]. However, in ciPTEC the SV40 large T antigen blockade is not absolute at 37 °C, some residual SV40 protein can be detected after the temperature switch. For *in-vitro* use, this immortalization procedure is very effective. However, for clinical application a more robust cell line would need to be developed. There are some other aspects that should be taken into account when using retrovirally-immortalized cells in a clinical application. Although retroviral vectors are engineered to be defective in their replication, replication competent retroviruses may be generated when recombination between the transfer vector, packaging components or endogenous retroviral elements [93,94]. All ciPTEC cell lines that were described in this thesis were tested for the presence of endogenous replication competent retroviruses and found to be negative (data not shown). Furthermore, the stable cell lines were unable to form viral particles. However, a serious point of concern regarding the use of these vector-infected cells in humans is the increased risk of recombination events due to subsequent virus infections within the patient or activation of endogenous retroviruses. To

prevent recombination, additional safety measures, such as co-transduction with a suicidal gene or further splitting of the viral genome, might be necessary [95].

Immunogenic effects

The introduction of foreign biological material to a human body will often be accompanied with the induction of an immune response by the host. In the case of organ transplantation, this process is recognized and well-studied [96]. With the application of immunosuppressants and careful matching of MHC complexes, it is possible to minimize this immune response and avert organ rejection [97]. In cell therapy, which is mostly based on the infusion of live marrow stromal cells (MSCs) into the circulation of the patient, the low antigenicity and immunomodulatory properties of MSCs allow allogeneic transplantation [98]. Whether the use of allogeneic methods would be feasible for production of an off-the-shelf RAD remains to be examined. Theoretically, it should be possible to culture endogenous renal cells from each patient that is to be treated with a bioartificial RAD. However, in practice, the number of cells necessary for a single or multiple devices, by far exceeds the yield from a renal biopsy or a urine sample, and would require extensive propagation [99]. In future, the use of induced pluripotent stem (iPS) cell technology might provide another option [100]. Not only can iPS cells propagate indefinitely given the right stimuli, they can also differentiate into other cell types in the human body. Various investigations are being performed to develop patient-specific iPS treatment, thereby reducing the risk of immunogenic effects [101-104].
However, the development and characterization of conditionally immortalized, or iPS cell lines for each single patient are costly and time consuming. Therefore, the general application of a single isolated and fully characterized ciPTEC line still seems to be a promising strategy. Nevertheless, it is important to take the safety aspects into account. While cultured in a hollow fiber based bioartificial RAD, the cells are separated from the circulation by a semi-permeable membrane. Particles smaller than the pore size of the membranes' molecular weight cut-off, such as immunoglobulin or soluble MHC complexes, might pass the membrane and elicit an immune response. Therefore, the immunogenic effects of applying ciPTEC in a bioartificial device need to be evaluated thoroughly, both *in-vitro* as well as *in-vivo*.

OTHER INNOVATIVE APPROACHES IN THE TREATMENT OF RENAL FAILURE AND UREMIA

Various approaches to remove uremic retention solutes in patients are being investigated by a number of research groups worldwide. These strategies mostly focus on a reduction in the intestinal uptake of uremic retention solutes, extract UTs directly from the circulation, or explore alternative ways of their elimination. The oral sorbent AST-120 was found to decrease serum indoxyl sulfate levels in both dialyzed and un-dialyzed patients with CKD, through

adsorption of indole, a precursor of indoxyl sulfate [105]. Clinical trials of AST-120 have indicated a beneficial effect on time to dialysis and a reduction of symptoms of uremia [106].

Brettschneider *et al.* recently reported the effective use of hydrophobic and cationic adsorbers for the extracorporeal removal of protein-bound, hydrophobic UTs. This system proved more effective in the removal of phenylacetic acid, indoxyl sulfate and p-cresyl sulfate then conventional hemodialysis techniques [107]. A combination of these absorbers together with conventional hemodialysis might provide easily implemented therapeutic options to increase the removal rate of these UTs. Another example is the wearable artificial kidney (WAK) [108-110]. In pilot studies with ESRD patients this wearable hemodialysis device demonstrated solute clearance, including middle molecules, and effective ultrafiltration [111-113]. Adsorbent-based treatment options are viewed as very promising tools for the removal of uremic solutes. As no biological components in the form of cultured cells are involved, many complicating factors, like cellular expansion, transport storage and safety concerns, are taken out of the equation.

However, in a bioartificial RAD, living ciPTEC membranes would provide additional support in the removal of accumulating UTs together with the benefits of solute reabsorption, and could offer important metabolic, paracrine, endocrine, and immunologic signaling. In a recent study by Westover *et al.* [114], treatment with an extracorporeal device containing PTEC seeded porous disks showed to convey a clear survival advantage in a porcine model of septic shock. This bioartificial renal epithelial cell system (named BRECS) was perfused with ultrafiltered blood or body fluids, but did not provide active transepithelial clearance of uremic solutes. The amelioration of the effects of septic shock was ascribed to the immunoregulatory role of tubule cells [115,116]. These effects on the deranged inflammatory response in acute kidney injury AKI [117,118], were already described in initial (pre-) clinical studies by Humes *et al.* [119]. Although the effects are promising, it is still unclear which paracrine factor secreted by renal epithelial cells is responsible for the observed anti-inflammatory response.

PERSPECTIVES FOR FUTURE RESEARCH

The results of this thesis show that the development of a bioartificial device such as a RAD based on ciPTEC might be possible from a cell-biological point of view. By demonstrating the presence of functional (cationic) uremic toxin elimination pathways by 2D ciPTEC monolayers, the groundwork has been laid for the demonstration of active uremic solute removal through living membranes. At this time, however, we cannot estimate if the addition of a RAD would make renal replacement therapy more efficient. Nevertheless, innovation often leads to development of novel insights. Before making the translation to *in-vivo* testing of a device, there are several interesting and also crucial aspects that deserve future attention.

The translation of the flat PES membrane based set-up to a 3D hollow fiber environment, which can be subjected to flow, will involve experiments with regard to membrane coating and cell monolayer formation. With cells cultured on the inside of hollow fiber bioreactors, it will be challenging to prevent obstruction of both the fiber lumen as well as the pores, as a result of coating or cell seeding. Optimization strategies may entail the inclusion of bioactive or ECM peptides into the hollow fiber material itself, thereby eliminating the need for coating. Furthermore, seeding cells under flow conditions could prevent clogging of the system during cellular expansion. In this regard, the use of a bioreactor, a closed system in which the cells can be cultured under strictly defined conditions, provides good opportunities to control pressure, flow, and could provide normoxic (renal) conditions. This latter factor cannot be achieved in "open" cell culture where oxygen levels reach up to 21%, which could affect cellular functionality and proliferative capacity [120,121]. Furthermore, investigations towards both the short and long term efficiency of uremic solute removal under bovine serum-free culture conditions are indispensable in the preparation towards a (pre-) clinical application.

Regarding the cell model, the absence of OAT1 and OAT3 expression limit the eventual clearance capacity of a RAD. As indicated before, endogenous expression of these transporters has not yet been achieved. In our lab, first steps have been undertaken for the introduction of these transporters through overexpression (unpublished data). Artificial introduction implies further genetic modification of the cell model, which complicates the use of this model in a clinical setting. A one to one comparison between the uremic solute clearance capacities of the existing ciPTEC cell line to OAT1/3 (over)expressing ciPTEC can provide important insights in the role of these OATs in toxin clearance.

In the evaluation of transmembrane toxin clearance within a bioreactor or a RAD, sophisticated bioanalysis methods that utilize LC-MS/MS provide opportunities to directly measure toxin levels [62-64]. This is preferred, as it circumvents the indirect estimation of transport processes by using radiolabeled or fluorescent model compounds such as [^{14}C]-labeled creatinine or ASP^{+}. Furthermore, these analysis techniques can be applied in *in-vivo* settings where the detection of UTs in plasma has to take place in complex biological mixtures. In this respect, animal investigations will be a necessary step for the investigation of the clinical feasibility, proof-of-principle and safety of a RAD. Evaluation will involve testing of the immunogenic and oncogenic properties of the living membranes and transport functionality of ciPTEC.

The results within this thesis expanded our knowledge on uremic toxin handling by proximal tubular monolayers, and provide ample ground for further research and development towards the *in-vivo* application of a bioartificial device in the treatment of uremia related pathologies.

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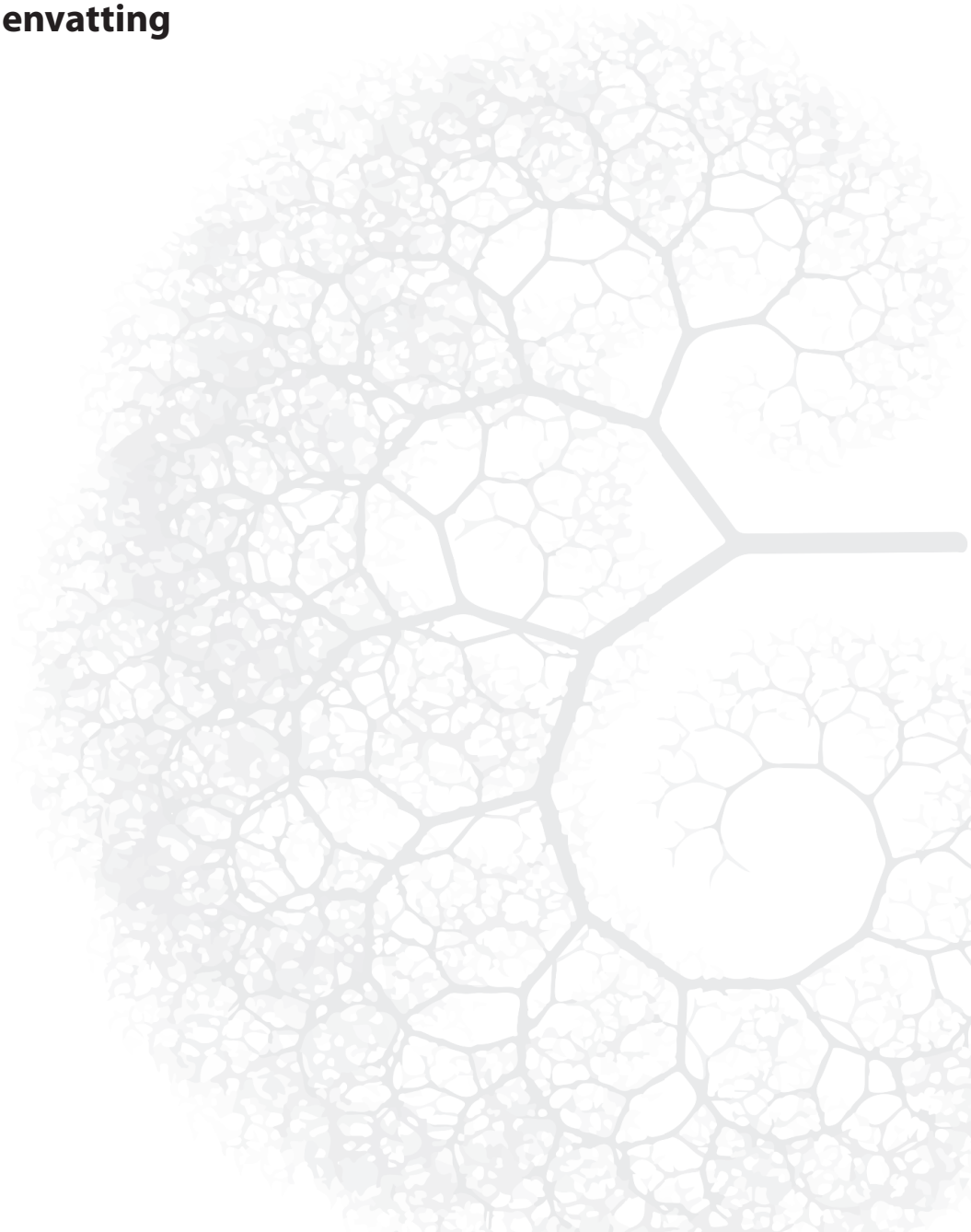
8

Summary
Samenvatting



SUMMARY

In patients suffering from Chronic Kidney Disease (CKD) or End-Stage Renal Disease (ESRD), kidney function is severely impaired. As a consequence, waste products are insufficiently cleared from the circulation. The systemic accumulation of these retention solutes contributes to the development of uremic syndrome, which is associated with increased morbidity and mortality. Over the last decade, more than 150 uremic retention solutes have been identified that accumulate during renal failure. Many of these solutes have the potential to act as uremic toxins, because they exert adverse biological or biochemical activities. Dialysis therapy aims to remove as many of these solutes as possible, thereby hoping to alleviate their destructive effects. Although current dialysis therapy can prolong many ESRD patients' lives, the treatment is unable to replace renal function completely. Mortality (15-20% per year) and morbidity of these patients remain high, whereas their quality of life is generally low. Renal replacement therapy removes mainly small, unbound substances from the circulation, while leaving large, compartmentalized and protein-bound uremic retention solutes untouched.

The need for improved renal replacement therapy has stimulated research into the development of a so-called bioartificial kidney or renal assist devices (RAD). These innovative devices aim to complement the hemodialysis treatment, by coupling artificial membranes with functional kidney cells. In the scope of this thesis, we examined the handling of cationic uremic toxins by proximal tubule epithelial cells (PTEC). In the kidney, PTEC play an active role in the clearance of organic solutes and uremic retention solutes. By focusing on the *in-vitro* epithelial transport pathways, and regulation involved in the renal removal of cationic uremic retention solutes, we aim to elucidate the possibilities to utilize living renal epithelial PTEC membranes for the improvement of hemodialysis therapy.

To study the function of renal cells *in-vitro*, the functionality of cell models has to reflect the native situation. It is therefore essential to characterize the nephron-specific properties, such as polarized transport, (re)uptake of solutes, and the formation of tight monolayers, in both established and novel cell lines. In **Chapter 2**, we compared the functional properties of two newly developed conditionally immortalized PTEC cell lines (ciPTEC) originating from kidney tissue to ciPTEC previously isolated from urine. The presence and functionality of proximal tubule specific transporters, like the influx carrier Organic Cation Transporter 2 (OCT-2), the efflux pumps P-glycoprotein (P-gp), Multidrug Resistance Protein 4 (MRP4) and Breast Cancer Resistance Protein (BCRP), was confirmed with only slight differences between the three cell lines. Extracellular matrix (ECM) genes collagen I and -IV α1 were highly present in both tissue derived ciPTEC, whereas ciPTEC isolated from urine showed a more pronounced fibronectin I and laminin 5 gene expression. Furthermore, functional endogenous albumin and phosphate reabsorption was observed in all models. The presence of these endogenous and specific proximal tubular features marks these cell lines as promising tools to study the uptake and elimination pathways of uremic solutes by PTEC. As a first

step in examining these clearance processes, **Chapter 3** focuses on the interaction of cationic uremic toxins with organic cation influx carriers (OCTs) in ciPTEC. OCT transporter activity was measured and validated in cell suspensions by studying uptake of the fluorescent substrate 4-(4-(dimethylamino)styryl)-N-methylpyridinium-iodide (ASP^+). Subsequently, the inhibitory potencies of the cationic uremic toxins, cadaverine, putrescine, spermine and spermidine (polyamines), acrolein (polyamine breakdown product), guanidine and methylguanidine (guanidino compounds) were determined. Inhibition of cellular cation uptake was demonstrated for all uremic toxins, with acrolein showing the most potent interaction.

The accumulation of most uremic retention solutes is generally considered a result of a disturbance in their renal excretion. Next to direct inhibition of transporters, the toxic or regulatory effects of these compounds could play important roles in disease progression. **Chapter 4** illustrates that exposure of ciPTEC to various cationic uremic toxins can lead to release of inflammatory cytokines IL-6 and IL-8, which are correlated with increased mortality and poor disease outcome in renal failure. Furthermore, an increase in endothelin-1 (ET-1) release was measured, which in renal failure is associated to interstitial fibrosis, proteinuria, cardiomyopathy and glomerulosclerosis. Exposure of ciPTEC to ET-1 was found to differentially regulate the functionality of OCT-2, through the ET-1 endothelin-β receptor axis. This led to a down-regulation of organic cation uptake after 30 minutes, while long-term exposure increased ASP^+ uptake significantly. These findings indicate that uremic solutes are able to influence transport processes not only by (competitive) inhibition, but also through secondary regulatory pathways.

Influx transport of uremic toxins is a crucial first step in the elimination of (uremic) solutes from the circulation. However, for the development of a bioartificial kidney, or RAD, the combination of functional *in-vitro* influx and efflux transport is essential to facilitate clearance. Therefore in **Chapter 5** we focused on the transepithelial transport processes in living ciPTEC grown on artificial semi-permeable surfaces. We have shown that ciPTEC can grow to a confluent monolayer on commercially available polyester Transwell® membranes. However for clinical application the use of hemocompatible materials would be preferred. Therefore, we optimized a coating consisting of 3,4-dihydroxy-L-phenylalanine (L-DOPA) and collagen IV that enables ciPTEC to be cultured to a confluent monolayer on polyethersulfone (PES) dialysis membranes. By optimizing the coating protocol, we were able to maintain water permeability of the membrane and promoting cell adhesion, while blocking vital blood components. Using immunocytochemistry, tight junctions could be detected. Furthermore, as an important step towards the development of a RAD, the functionality of vital clearance processes was shown by transepithelial transport of radiolabeled creatinine.

For ciPTEC characterization, static culture conditions were used. However, perfusion plays an important role both in the *in-vivo* situation as well and in a biological RAD. Tissue perfusion allows delivering nutrients and soluble factors to cells, but also the removal of metabolic waste relevant for homeostasis. **Chapter 6** describes the effect of fluid flow on transepithelial

cation transport by ciPTEC. We evaluated the effects of flow on monolayer quality as well as the transepithelial creatinine transport. The confluent monolayers were either cultured statically, or on a 2d plate rocker. Fluid movement was found to increase epithelial characteristics such as cilia length and monolayer height. Furthermore, the mRNA expression levels of the organic cation transporting influx and efflux transporters examined (*viz.* OATP4C1, OCT1, OCT2 and MATE1, MATE2K) increased significantly. This was accompanied by higher transepithelial flux of radiolabeled creatinine, as well as increased cellular sensitivity to cationic uremic toxins.

The increased transport observed under dynamic culture conditions indicates that the use of a relatively simple system for modeling fluid flow positively affects cell functionality. Enhancing the endogenous cellular features of ciPTECs, could make this cell line even more interesting for research towards renal clearance processes in pharmacological and physiological studies. Furthermore, transepithelial solute transport is an important aspect of an innovative bioartificial RAD.

In this thesis we aimed to functionally characterize uremic solute handling by ciPTEC, by evaluating the *in-vitro* functional characteristics of cultured ciPTEC monolayers and measuring their organic cation transport properties. In **Chapter 7,** the implications of the results are considered and crucial considerations important for future research into the development of a RAD are being discussed. Our results showed that the stable ciPTEC model is a promising candidate for the development of living membranes to use in a RAD. However, before such a concept can be applied in an animal or clinical setting, further research should focus on the existing challenges regarding the design, 3D engineering, its functionality, durability, associated safety concerns and costs.

SAMENVATTING

Patiënten die lijden aan chronisch of eindstadium nierfalen hebben een ernstig verstoorde nierfunctie. Als gevolg hiervan worden afvalstoffen onvoldoende verwijderd uit het bloed. De stapeling van deze zogenoemde uremische toxines (UTs), kan bijdragen aan de ontwikkeling van het uremisch syndroom, dat geassocieerd is met een verhoogde kans op de ontwikkeling van gerelateerde ziekten en op overlijden. In de afgelopen tien jaar zijn er meer dan 150 uremische afvalstoffen geïdentificeerd die zich ophopen tijdens nierfalen. Veel van deze opgeloste stoffen hebben een nadelig effect op biologische en biochemische lichaamsprocessen. De behandeling van nierpatiënten met (hemo)dialyse is er dan ook op gericht om zoveel mogelijk van deze afvalstoffen te verwijderen. Hoewel de huidige dialysetechnieken het leven van veel nierpatiënten verlengt, kan de behandeling de nierfunctie helaas niet voldoende overnemen. Onder nierpatiënten die behandeld worden met dialyse blijft de mortaliteit hoog (15-20% per jaar), terwijl de kwaliteit van leven relatief laag is. Een van de mogelijke redenen hiervoor is dat nierfunctievervangende therapie voornamelijk kleine, niet-eiwitgebonden stoffen uit de circulatie verwijdert. De uitscheiding van grote, complexe en eiwitgebonden UTs is onvoldoende, waardoor deze afvalstoffen blijven stapelen in het bloed met allerlei bijkomende problemen, zoals vaat- en hartlijden.

De behoefte aan betere nierfunctievervangende therapie gaf de aanleiding voor het onderzoek naar de ontwikkeling van een biologische kunstnier, of een renal assist device (RAD). Door gebruik te maken van "levende membranen", bestaande uit niercellen gekweekt op polymeren, zouden UTs op een efficiëntere manier verwijderd kunnen worden. In dit proefschrift onderzochten wij hoe kationische (positief geladen) UTs worden getransporteerd door proximale tubulus-epitheelcellen (PTEC). In de nier zijn deze cellen namelijk van groot belang voor het transport van organische afvalstoffen. We hebben de processen en mechanismen die betrokken zijn bij het transport van kationische organische stoffen onderzocht om zo meer inzicht te krijgen in de mogelijkheden om levende PTEC-membranen toe te passen in verbeterde nierfunctievervangende therapieën.

Om de functie van niercellen *in vitro* te kunnen bestuderen, is het van belang dat de gebruikte celmodellen functionele eigenschappen bezitten die oorspronkelijk in de nier aanwezig zijn. Nieuwe cellijnen worden daarom gecontroleerd op specifieke kenmerken, zoals de vorming van dichte celmonolagen, directioneel transport, en (her)opname van opgeloste stoffen. In **hoofdstuk 2** hebben we de eigenschappen van twee, uit humaan nierweefsel ontwikkelde, conditioneel geïmmortaliseerde PTEC cellijnen (ciPTEC) vergeleken met een bestaande ciPTEC lijn ontwikkeld uit humane urine. Het grootste verschil tussen de cellijnen werd gevonden in het vermogen om biologische weefselstructuren te produceren. In de cellijnen ontwikkeld uit nierweefsel, was het RNA coderend voor weefselstructuren zoals collageen I en collageen IV duidelijk aanwezig. In de oorspronkelijk uit urine ontwikkelde ciPTEC werd juist meer fibronectine I en laminine 5 gevonden. Dit zou kunnen betekenen

dat cellen geïsoleerd uit urine het vermogen om hun extracellulaire matrix aan te maken deels hebben verloren. Desalniettemin kon de functionele aanwezigheid van meerdere specifieke transporteiwitten, zoals de organische-kation transporter 2 (OCT2), P-glycoprotein (P-gp), multidrugresistentie-eiwit 4 (MRP4) en breast cancer resistance protein (BCRP), worden bevestigd in alle de drie onderzochte cellijnen. Ook waren de cellijnen in staat tot (her) opname van fosfaat en albumine, belangrijke eigenschappen van de proximale tubulus. De aanwezigheid van deze specifieke kenmerken maakt de ciPTEC een veelbelovend celmodel om de uitscheidingsprocessen van UTs nader te bestuderen.

Hoofdstuk 3 richtte zich vervolgens op de interactie tussen kationische UTs en organische-kation transporters (OCTs) in ciPTEC. De geteste UTs, cadaverine, putrescine, spermine en spermidine (polyamines), acrolein (polyamine afbraakproduct), guanidine en methylguanidine (guanidines), hadden allemaal een remmend effect op de opname van de fluorescente stof 4-(4-(dimethylamino)styryl)-N-methylpyridinium-iodide (ASP^+). Acrolein was hierin het meest potent. Deze bevindingen geven aan dat UTs een competitieve/concurrerende interactie aangaan met de transporters. De stapeling van de meeste UTs wordt gezien als het gevolg van een verstoorde uitscheiding. Zoals aangetoond in Hoofdstuk 3, kan deze verstoring veroorzaakt worden door directe remming. Daarnaast zouden uremische afvalstoffen transportprocessen kunnen beïnvloeden door hun toxische of regulerende effecten.

In **Hoofdstuk 4** hebben we laten zien dat blootstelling van ciPTEC aan verschillende kationische UTs leidt tot de afgifte van de ontstekingsfactoren IL-6 en IL-8. Deze ontstekingsfactoren worden vaak gezien bij chronische ontstekingsprocessen, en worden in verband gebracht met een slechte overleving bij nierfalen. Ook zorgt de blootstelling van ciPTEC aan UTs voor een verhoogde afgifte van het bloeddrukregulerende hormoon endotheline-1 (ET-1). Verhoogde ET-1 concentraties in het bloed worden in verband gebracht met de ontwikkeling van chronisch nierlijden. De bevindingen beschreven in Hoofdstuk 4 tonen daarnaast ook aan dat ET-1 de OCT gemedieerde opname van ASP^+ door ciPTEC kan reguleren. Na 30 minuten blootstelling was de opname van ASP^+ verminderd, terwijl ET-1 op langere termijn (24 uur) leidde tot een toename in ASP^+-opname. Deze bevindingen wijzen erop dat het transport van UTs niet alleen door middel van competitieve remming kan worden beïnvloed, maar dat andere regulerende mechanismen, zoals ontstekingsprocessen, ook een rol kunnen spelen.

De opname van uremische toxines in de niercel is een cruciale eerste stap in de uitscheiding van (uremische) afvalstoffen. Maar voor de ontwikkeling van een biologische kunstnier of RAD is het ook van belang dat uitscheidingsprocessen kunnen plaatsvinden in het gebruikte celmodel. In **Hoofdstuk 5** hebben we ons daarom gericht op het meten van transportprocessen door ciPTEC-cellagen gekweekt op kunstmatige membranen. We hebben aangetoond dat ciPTEC mooie dichte cellagen vormen op, commercieel verkrijgbare, polyester Transwell® membranen en polyethersulfon (PES) dialysemembranen. Omdat de PES-membranen zijn ontworpen om in contact te staan met bloed, zijn ze klinisch beter toepasbaar, maar gaan ze celhechting ook grotendeels tegen. Om toch dichte celmonolagen te kunnen kweken, hebben

we het membraan gecoat met 3,4-dihydroxy-L-fenylalanine (L-DOPA) en collageen IV. Door gebruik te maken van deze geoptimaliseerde coating, bleef de waterdoorlaatbaarheid van het membraan behouden terwijl de vorming van een cellaag werd gestimuleerd. Bovendien waren de cellen in staat om creatinine over het membraan te transporteren, wat impliceert dat belangrijke transportprocessen functioneel behouden blijven.

Tijdens de *in vitro* kweek en karakterisatie van ciPTEC zijn voornamelijk statische kweekomstandigheden gebruikt. Echter, in de gezonde nier worden tubulus cellen continu blootgesteld aan de stroming van voorurine of bloedplasma. Ook in een biologische kunstnier zal vloeistofstroming een belangrijke rol spelen. In **Hoofdstuk 6** is daarom het effect van stroming op het organisch-kation-transport en de kwaliteit van de cellaag onderzocht door de cellen te kweken op een 2D kantelplaat. De beweging van vloeistof over de cellen zorgde voor een toename in het epitheliale karakter van ciPTEC, en ging gepaard met een verhoogd transport van creatinine. Daarnaast bleken de cellen gevoeliger voor de toxische effecten van kationische UTs. Deze resultaten geven aan dat de simulatie van vloeistofstroming tijdens de kweek van celmonolagen de celfunctionaliteit en gevoeligheid van ciPTEC zou kunnen verbeteren.

Samenvattend kunnen we stellen dat in dit proefschrift het transport van organische kationen door ciPTEC is gekarakteriseerd, waarmee inzicht is verkregen in de mogelijkheden om dit celmodel toe te passen in toekomstige nierfunctievervangende therapieën. **Hoofdstuk 7** bediscussieerde de implicaties van deze resultaten. Het ciPTEC-model blijkt veelbelovend voor de ontwikkeling van een biologische kunstnier of RAD. Toch zal verder onderzoek nodig zijn om inzichten te verkrijgen in de toepasbaarheid van het ontwerp, het gebruik van holle vezels, de functionaliteit, duurzaamheid, veiligheid en de kosten, voordat de biologische kunstnier in een dierexperimentele of klinische setting zal kunnen worden getest.

9

Dankwoord
List of abbreviations
Curriculum Vitae
Publications

DANKWOORD

Toen ik in 2010 begon aan dit project had ik nooit gedacht dat er zoveel mensen betrokken zouden zijn bij het tot stand komen van dit boekje. Wat fijn dat ik jullie nu eindelijk allemaal mag bedanken voor de hulp, de inspiratie, de ondersteuning en de gezelligheid, die de afgelopen jaren een grote rol hebben gespeeld gedurende mijn promotietraject.

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Linda en Mireille, jullie begrijpen als geen ander wat er komt kijken bij een promotieproject, maar dan aan de UvA, VU of UCL. Stuk voor stuk bewonder ik jullie doorzettingsvermogen en ik ben blij dat we ondanks ons drukke schema nog regelmatig leuke dingen doen. Jacqueline, Nicolien en Mariska, bedankt voor de gezelligheid van al die keren dat we afspraken in de kantine van het Radboud. Onder het genot van een boterhammetje kwamen we tot "rust" en lieten we onze dagelijkse bezigheden de revu passeren. Jullie steun en adviezen hebben me vaak genoeg uit een dipje geholpen!

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
AG	Aminoguanidine
ASP	4-(4-(dimethylamino)styryl)-N-methylpyridinium-iodide
ATP	Adenosine-Tri-phosphate
ATR-FTIR	Attenuated Total Reflection-Fourier Transform Infrared Spectra
BCRP	Breast Cancer Resistance Protein
BioKid	Bioartificial Kidney (Renal Assist Device)
BRECS	Bioartificial Renal Epithelial Cell System
BSA	Bovine Serum Albumin
cGMP	Cyclic Guanosine Monophosphate.
CiPTEC	Conditionally Immortalized Proximal Tubule Epithelial Cell
CiPTEC-T1	Conditionally Immortalized Proximal Tubule Epithelial Cell, Tissue Origin 1
CiPTEC-T2	Conditionally Immortalized Proximal Tubule Epithelial Cell, Tissue Origin 2
CKD	Chronic Kidney Disease
CN	Normal Concentration
Coll IV	Collagen IV
CU	Uremic Concentration
DAG	Diacylglycerol
DOG	Sn-1,2-dioctanoyl glycerol
ECM	Extra Cellular Matrix
EDS–EDAX	Energy Dispersive Spectroscopy analysis
EMT	Epithelial to Mesenchymal Transition
ESRD	End Stage Renal Disease
ET-1	Endothelin-1
EUtox	European Uremic Toxin (Work Group)
FCS	Fetal Calf Serum
G418	Geneticin
GFR	Glomerular Filtration Rate
HBSS	Hanks' balanced salt solution
HD	Hemodialysis
HDF	Hemodiafiltration
HEK	Human Embryonic Kidney
HF	High Flux
hTERT	Essential catalytic subunit of human telomerase
IC50	Half maximal Inhibitory Concentration
IgG	Immunoglobulin G

IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
J	Flux
JTC-12	Monkey Kidney Proximal Tubule cell line
Ki	Dissociation Constant
L-DOPA	3,4-dihydroxy-L-phenylalanine
LLC-PK	Lilly Laboratories Cell Porcine Kidney proximal tubular cells
L-NMMA	N(G)-monomethyl-l-arginine
LPS	Lipopolysaccharide
MATE	Multi-antimicrobial extrusion protein
MDCK	Madin-Darby Canine Kidney Cells
MF	Microfiltration
MRP2	Multidrug Resistance Protein
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NaDC3	Na^+-dicarboxylate cotransporter
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OA	Organic Anion
OAT	Organic Anion Transporter
OATP	Organic Anion Transport Protein
OC	Organic Cation
OCT	Organic Cation Transporter
OCTN	Carnitine/Organic Cation Transporter
PAN	Polyacrylonitrile
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline - Tween
PDA	Adherent Polydopamine
PES	Polyethersulfone
PSF	Polysulfone
PTEC	Proximal Tubule Epithelial Cell
RAD	Renal Assist Device
RAP	Receptor-associated protein
RO	Reverse Osmosis
SC	Sieving Coefficient
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SEM image	Scanning Electron Microscope Image
SLC	Solute Carrier

SV40-T	Simian Virus 40 large T antigen
TEER	Trans-Epithelial Electric Resistance
TPA	Tetrapentylammoniumchloride
URAT	Urate Reuptake Transporter
UT(s)	Uremic Toxin(s)
V/V	Volume/Volume
W/V	Weight/Volume
WAK	Wearable Artificial Kidney
ZO-1	Zona Occludens 1

CURRICULUM VITAE

Carolien Schophuizen werd geboren op 11 september 1987 te Eindhoven. In 2005 behaalde zij haar VWO-diploma aan het Pleincollege Eckart. In datzelfde jaar begon ze aan de opleiding Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Haar bachelorstage liep ze bij de afdeling Farmacologie & Toxicologie van het UMC St. Radboud in het Nijmegen Centre For Molecular Life Sciences (NCMLS), onder leiding van Prof. Dr. Frans Russel, Dr. Jan Koenderink en Dr. Reginald Kavishe, alwaar zij onderzoek deed naar de expressie van Plasmodium Falciparum ABC transporters in HeLa en Sf9 cellen. Ook was Carolien betrokken bij de evaluatie van het curriculum als studentvoorzitter van de opleidingscommissie Biomedische Wetenschappen. Ze vervolgde haar studie met de master Biomedical Sciences, en richtte zich op de hoofdvakken Toxicologie en Pathobiologie. Tijdens haar eerste masterstage verrichtte zij onder leiding van Prof. Dr. Tinka Murk en Dr. Jaime Freitas onderzoek op de afdeling Voedingsmiddelentoxicologie van de Wageningen Universiteit. Haar tweede masterstage vond plaats aan het Karolinska Institutet in Zweden, op de afdeling Biochemical Toxicology van het Institute of Environmental Medicine. Onder de begeleiding van Prof. Dr. Ralf Morgenstern en Dr. Katarina Johansson, en mede dankzij een subsidie van KWF kankerbestrijding, bestudeerde ze daar de cytotoxiciteit van Doxorubicine en Actinomycine D derivaten, in MCF-7 en V79 cellen die Glutation-S-Transferases tot overexpressie brengen. Dit onderzoek resulteerde tevens in haar eerste publicatie als co-auteur.

In 2010 behaalde Carolien haar masterdiploma "cum laude", en gedurende dat jaar startte ze ook haar promotieonderzoek bij de afdeling Kindernefrologie van het Radboudumc in Nijmegen. Onder leiding van haar drie promotoren: Prof. Dr. L.P. van den Heuvel, Prof. Dr. Roos Masereeuw, en Prof. Dr. Joost Hoenderop, verrichtte ze onderzoek naar de opname en eliminatie van kationische uremische toxines door proximale tubulus cellen. Dit project (BioKid P3.01) maakte onderdeel uit van het onderzoeksprogramma van het BioMedical Materials Institute, mede gefinancierd door het ministerie van economische zaken en ondersteund door de Nierstichting. Carolien heeft meerdere studenten begeleid en haar werk op nationale en internationale congressen gepresenteerd. Haar onderzoek resulteerde in verschillende gepubliceerde artikelen en dit proefschrift. Gedurende haar promotietraject heeft ze tevens de postdoctorale opleiding toxicologie afgerond. Carolien is momenteel werkzaam als Regulatory Affairs Manager bij het Nijmeegse farmaciebedrijf Synthon BV.

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